

How to boost the careers of women in science?

This week, *Nature* launches an international web debate on the factors that lead to the scarcity of women in research. Tackling discrimination is a high priority.

Why is the rarity of women in science, especially at senior levels, a problem? A common response is that societies demanding a skilled technical workforce must stop squandering half of their scientific potential. This argument is being increasingly used by governments that wish to develop knowledge-based economies. A second aspect of the problem is the widespread frustration that is experienced by women who, for one reason or another, find their scientific potential difficult, if not impossible, to fulfil.

A third, more controversial, argument is that having more women scientists would lead to a broader range of research being tackled. Some concerned with the needs of developing countries, for example, argue that more women scientists would mean a greater emphasis on tackling societal challenges through research. Whilst one cannot be sure of the validity of this statement, it is reasonable to suggest that, to some degree, progress in science will reflect the backgrounds and interests of those doing it. We cannot and should not seek to legislate in order to have science fairly represent the needs of society, but society can reasonably expect that the make-up of the scientific community reflects that of society at large to a greater extent.

Of course, one needs to take into account not only the impact of childbirth, but also the fact that child-rearing is, in practice, still undertaken predominantly by women. Reconciling the needs of families and of scientific careers is something that industry appears to have tackled with more gusto than has academia. But there are other factors disadvantaging women that are much less widely acknowledged, as witnessed by consistent reports and peer-reviewed research from around the world showing variously that women scientists earn less, have less prestige within departments, have less lab space, get worse jobs on graduating with science

degrees, have more teaching responsibilities, have more difficulty getting grants and apply for fewer grants in the first place.

These and other issues will be described and discussed in a web debate launched by *Nature* today (<http://helix.nature.com/debates>). Contributors to the debate will discuss discrimination at the Massachusetts Institute of Technology (MIT), the problems faced by women researchers in Germany (and the women-only positions scheme), how different national governments and agencies are attempting to improve the situation, and the challenge of the issue at a European level and in the Third World. Since announcing the launch of the debate more than a week ago to networks interested in this issue, *Nature* has been deluged with e-mails from around the world from those wanting to follow it or to contribute. These e-mails, the tip of an iceberg, are testament to the continuing importance and relevance of this topic. The debate comes against a backdrop of continuing concern and increasing political activity (see *Nature* **400**, 195; 1999).

Some will argue that we must still wait for a trickle-up effect. But as Mary-Lou Pardue and colleagues eloquently argue in the debate about their department at MIT: "each generation of young women, including those who are currently senior faculty, began by believing that gender discrimination was 'solved' in the previous generation and would not touch them. Gradually, however, their eyes were opened to the realization that the playing-field is not level after all, and that they had paid a high price both personally and professionally as a result." The absence of significant progress, even in areas of science where consistently more than 50 per cent of graduates are female, suggests that continuing to preach a 'rewards-tomorrow' philosophy is not realistic. And discrimination is one key obstacle to be tackled by employers of women researchers and, as a source of additional pressure, by those who fund the employers. We invite readers to contribute to the debate by proposing actions. ■

Emergency as opportunity

Powerful new leadership at France's troubled Natural History Museum should benefit organismal biology.

It is not every day that a major research institute has control taken out of its hands by a government. Much less in France, where scientists usually take to the streets at even the smell of state 'interference'. Yet a *coup d'état* at Paris's Natural History Museum, where the science ministry has imposed an interim administrator — Jean-Claude Moreno — to run the centre as he (and he alone) sees fit, has not only been accepted, but has been generally welcomed by museum staff (see page 104).

Moreno brings with him a government pledge to spend FF2.6 billion (US\$420 million) to renovate what is one of the world's great museums, but also one of its most under-resourced and run down. Even the most politically naive scientists will appreciate this is not the moment to take up arms.

This large investment in the basic infrastructure of biodiversity

research is excellent news, as is the commitment it represents to whole-organism biology in the face of the siren calls of molecular biology. Natural history museums have a major role to play in creating a detailed picture of the Earth's biota, and managing it responsibly (see *Nature* **394**, 105; 1998).

Moreno's honeymoon will be short, however. With a string of multi-million-dollar national building projects behind him — including the museum's Grande Galerie — no one doubts his managerial competence. But Moreno, a non-scientist, himself admits that the more difficult task will be to reconcile the museum's missions of research, educating the public and curating collections in a dynamic whole. He seems committed to listen and to learn what scientists have to say. Museum researchers in France and elsewhere would do well to speak up. ■





US budget cuts
Clinton's chief of staff
slams Congress
plans for science
p103



Nanobacteria
A new form of life,
a mistake or a
case of fraud?
p105



New Zealand
Science leaps up the
government's
campaign agenda
p106



GM crops
US farmers are
worried that no one
wants their harvest
p107

US energy secretary roasts tardy nuclear weapons laser project

Washington

The National Ignition Facility (NIF), the \$1.5 billion centrepiece of the US Department of Energy's stewardship programme for the country's nuclear weapons stockpile, is running two years late and hundreds of millions of dollars over budget.

Bill Richardson, the energy secretary, revealed the cost overruns last week, in a blistering statement blaming the Lawrence Livermore National Laboratory and the University of California, which manages the laboratory for the Department of Energy (DoE), for letting them occur.



Step on it: project is two years behind.

Michael Campbell, the Livermore Laboratory's associate director of lasers and the director of the project, resigned on 27 August.

Campbell left after an anonymous tip-off that he had never received his PhD from Princeton University.

A crisis meeting on 31 August between DoE officials led by Gil Weigand, head of the nuclear weapons research programme, and Livermore Laboratory officials, revealed the extent of the problems, and led to Richardson's announcement on 3 September.

"I am deeply disturbed to learn of projected cost overruns and scheduling delays associated with the National Ignition Facility project," said Richardson. "I am also gravely disappointed with the University of California and the Department of Energy's Lawrence Livermore Laboratory for its late reporting of these significant problems."

Richardson announced a six-step plan to get the project "back on track," starting with an order to the Livermore Laboratory to subcontract out assembly of the NIF's giant laser systems, and the appointment of an outside panel to select "the best technical course of action" for the project.

The Livermore Laboratory issued a statement acknowledging "the importance of project management," but did not endorse Richardson's view that the project is over budget.

Laboratory officials believe that technical uncertainties have always threatened the project's timetable and budget — especially over the need to assemble it all in a clean-room atmosphere, and the challenge of getting the high-powered lasers to work. Critics of the NIF, led by the Natural Resources Defense Council in Washington, have long claimed that the machine is unlikely to meet its goal of igniting a fusion target.

A senior energy department official said that the NIF didn't face any major technical obstacles, but that the project planning had been mismanaged, and that the Livermore Laboratory would be unable to assemble the huge lasers itself. Giving the job to an outside contractor will prolong it, he said. The NIF would be ready in 2005 instead of 2003, increasing the cost of the project by "hundreds of millions of dollars". The outside panel, he said, would consider options including scaling back the project, reducing the number of lasers from 192 to 96 or delivering an energy of 1.5 megajoules (MJ) instead of the 1.8 MJ originally envisaged.

A source close to the laboratory said there were problems with the high-speed crystallization process used for making key optical

elements of the NIF. But the DoE official said he had no knowledge of such problems, and Richardson's statement said "the problems with the NIF are not technological: these are project management issues".

Richardson said that any cost overruns would come out of the Livermore Laboratory's \$1 billion annual budget, "so that the US taxpayer does not foot additional bills because of these problems". He promised to withhold "at least \$2 million" of the University of California's management fee for running the laboratory, and has asked the laboratory to discipline staff who may have covered up the problems. As recently as June, the laboratory said the project was on time and on budget (see *Nature* **399**, 622; 1999).

This is a bad time for the DoE and nuclear weapons laboratories. The department is facing difficulties with its other main scientific project — the Advanced Neutron Spallation Source at Oak Ridge National Laboratory in Tennessee. And the nuclear weapons laboratories, which are usually immune to political opposition, have been attacked for security lapses following Chinese espionage, leading some in Congress to question their \$4 billion annual budget. **Colin Macilwain**

Chimp care causes furrowed brows

San Diego

The United States' largest chimpanzee research facility has reached a deal with the government to settle a long-running dispute over allegations of substandard care.

The Coulston Foundation, in New Mexico, will reduce the number of chimps it houses by 300 over the next two years. It will bring in an outside review team to examine animal care and increase veterinary services.

A \$100,000 fine by the US Department of Agriculture (USDA) is being held in abeyance to ensure that the foundation complies with the settlement's consent agreement, under which it admitted no wrongdoing.

Questions remain about where the 300 chimps will go, where the money will come

from to pay for their care — the animals may live to the age of 50 — and how they may be used for future research.

Animal rights activists, who prompted the USDA action against the Coulston Foundation, have been pushing for sanctuaries for the research chimps, some of which have infections such as HIV.

Killing surplus chimps has been ruled out by the National Institutes of Health (NIH), which is reviewing proposals for long-term care of primates now kept at Coulston and at other facilities. A decision is expected by next spring.

About \$4 million a year is being set aside for their maintenance, but critics question whether that is enough. **Rex Dalton**

Japanese plan speeds up rice genome sequencing

Tokyo

An international project to sequence the rice genome looks likely to adopt a new goal, following an announcement by the Japanese government that it plans to sequence the entire rice genome by 2004.

The plan is yet to be incorporated into the new strategy of the 11-nation International Rice Genome Sequencing Project (IRGSP), which will meet in Thailand this month to discuss strategies.

The Ministry of Agriculture, Forestry and Fisheries (MAFF), which leads the Japanese project, says the decision to speed it up was motivated by intensifying competition from the private sector.

But even without such competition, researchers involved in IRGSP say that the pace of the project, which currently aims to sequence the rice genome by 2008, needs to be accelerated.

"The project's 10-year time scale was too long," says Takuji Sasaki, leader of MAFF's rice genome research programme, who says the new target can be achieved without significant strategic changes.

Sasaki says the project will keep to its plan to map expressed sequence tags (ESTs) of chromosomes 1, 5 and 6 for its initial phase, instead of an earlier proposal to map ESTs of the entire rice genome.

Members of IRGSP called for an accelerated effort when it was revealed in April that Celera Genomics, the US venture set up by geneticist Craig Venter, planned to sequence the entire rice genome using a whole-genome 'shotgun' sequencing technique (see *Nature* 398, 545; 1999).

MAFF also says it will step up support for rice-genome research as part of a government programme that promotes commercialization of biotechnology research (see *Nature* 397, 554; 1999).

In its budget proposal for the fiscal year 2000, the ministry asked for ¥7.4 billion (US\$67 million) for rice genome-related research, a three-fold increase from this year's budget, including ¥3.6 billion for sequencing the rice genome.

The budget proposal also includes a project to create a library of full-length rice complementary DNA, and functional genomics projects aimed at identifying and analysing the functions of the genes of economically significant plants.

These efforts will entail a collaboration with universities, industry and other national research institutes. This is seen as a major shift for MAFF, which has sought to conduct its rice-related research in its own laboratories.

Asako Saegusa

Physicists unite to combat a crisis of falling numbers

Paris

Presidents of Europe's physics societies met in the UK last week to discuss their growing concern over the lack of students and teachers entering the field.

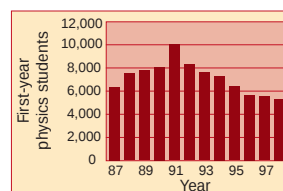
In Germany, the number of first-year physics students, which peaked at 10,000 in 1991, had fallen to just over 5,000 last year. In Britain, the number of physics graduates training to be schoolteachers collapsed from 568 in 1993 to 181 in 1998.

Similar examples can be seen in most European countries. "It is a crisis situation," says John Lewis, treasurer of the European Physical Society (EPS), which organized the meeting, at Malvern College in Worcestershire, of 50 physicists including 20 presidents of national physics societies.

The American Institute of Physics was also represented. The number of US students completing first degrees in physics is the lowest for 40 years, though the number of graduates in all subjects has quadrupled in that time.

Speakers blamed poor teaching at school and inadequate public understanding. "The image of physics is not good," Lewis says. "People associate it with nuclear disasters. They don't see how it can benefit people."

Sir Arnold Wolfendale, president of the EPS, points to a lack of well trained teachers and to the low salaries they receive. "Teachers



Let's get physical: no thanks, say German students.

of all sorts have slipped way behind the average pay scale," Wolfendale says. "Unless you get good teachers you don't turn the kids on."

Alexander Bradshaw, president of the German Physical Society, blames low pay for graduate students. "A trend that we have been noticing in several European countries is that physics students are reluctant to get PhDs or go into research," he says. "They are getting good offers from banks or insurance companies. We have benefited too long from the cheap labour of graduate students."

Participants now plan to take their case to national governments and to the European Union. Some have begun projects to promote the image of physics. The EPS is sponsoring a television series about the uses of physics and is producing videos for schools.

Aart Kleyn, president of the Netherlands Physical Society, says the meeting has brought public education to the forefront of issues being considered by physics groups. "These problems are as important as funding, if not more so," he says.

Heather McCabe

India urged to lift map restrictions

New Delhi

Under pressure from scientists and industry, the Indian government is to review its restrictions on the dissemination of geographical data collected by its satellites and agencies such as the Survey of India (SOI).

At present, high-resolution maps produced by SOI — the country's only map-maker — and satellite pictures showing military installations are not available to the public because of national security. And the use of aerial photography is banned unless directly authorized by the defence ministry.

"A lot of scientific and developmental work is suffering in India because of the non-availability of maps and geodetic survey data," says Madhav Kulkarni, a civil engineer at the Indian Institute of Technology in Mumbai.

Kulkarni was one of 500 participants at the MapIndia-99 conference held last week in New Delhi, which was organized by the Indian Space Research Organization and the Centre for Spatial Database Management and Solutions.

A. R. Das Gupta, deputy director of the Space Application Centre in Ahmedabad, said the centre cannot disseminate its high-resolution satellite data because of defence ministry restrictions.

Scientists say that the absence of easy access to 1:50,000-scale maps is placing a major impediment on their work without serving any real security function. One speaker said that there was no public access to 1:50,000-scale maps for more than 60 per cent of India's land mass.

Krishnaswami Kasturirangan, chairman of the Indian Space Research Organization, and Valangiman Ramamurthi, secretary of the science ministry, which controls SOI, both say the current policy should be reviewed.

But Ramamurthi says the government has yet to decide on what data restrictions to remove. Given India's security concerns, scientists expect some to stay. The conference called for "a balanced mapping policy that takes care of security concerns without hindering development projects".

K. S. Jayaraman

Clinton flails Congress over \$1.8 billion civilian R&D cuts

AP Washington

The Clinton administration has vigorously attacked Republican plans to cut research spending at science agencies, signalling its intention of portraying Congress as hostile to science, technology and innovation during budget negotiations this autumn.

John Podesta, President Bill Clinton's chief of staff, made science and technology the focal point of a high-profile address on budget issues at Washington's National Press Club on 1 September.

"This year, the Republican-led Congress, to make room for its risky tax plan, is playing politics with science and technology funding," said Podesta. He warned that the planned cuts were "threatening the potential progress of innovation in America".

Podesta said that the proposals so far amounted to a \$1.8 billion cut in civilian research and development funding, of which \$1 billion is at NASA (see below), while projects worth an additional \$1 billion had been allocated to specific institutions — "undermining peer review and slashing funding for higher-priority projects".

The attack is intended to put Republicans on the defensive as they return to Washington this week to complete work on the bill and on another, even more contentious, that will contain funding for the National Institutes of Health.

Science lobbyists are delighted by the high-profile defence of science and technology programmes, though it is unclear where the money will come from to restore their



Podesta: 'Congress is playing politics'.

James Sensenbrenner (Republican, Wisconsin), chair of the House Science Committee, said Clinton's plan "depends on budgetary tricks such as tax hikes and user fees that will never be enacted".

Congress allocated more to research and development than the administration had asked for in three of the past four years, Sensenbrenner added. "We hope the president's staff view science funding as a priority, not a short-lived political gimmick."

But Sensenbrenner faces embarrassment this month if, as seems likely, bills are passed that cut science programmes to levels well below those authorized earlier in the year by his committee. One lobbyist said Sensenbrenner "lacked friends" to help him defend the programmes under his jurisdiction.

President Clinton, too, lacks friends in Washington, but he knows a political opening when he sees one, and looks set to emerge with most of the credit if funding for the programmes is restored.

Colin Macilwain

funding. Clinton and Congress are committed to agree a budget that falls within tight caps agreed in 1997. Podesta told reporters that Clinton's original budget proposal, published in February, could fit within these limits — but few independent observers believe this is possible.

NASA could lose 'best-value space projects'

NASA Washington

The proposed \$1 billion cut to NASA's budget request is aimed at the small, focused science projects that are a hallmark of the agency's 'faster, better, cheaper' philosophy, observers say.

The committee "cut the programmes that had the least political constituency," says one congressional staffer. These include Discovery planetary missions, the Explorer programme and a line of low-cost Earth Probes.

The House appropriators cut \$265 million from the \$2.2 billion request for space science and \$301 million from the \$1.46 billion Earth-science request. Cuts in the Explorer programme would stop



Mission impossible? The Pluto-Kuiper Express is under threat.

NASA initiating new small and medium-sized missions in space physics and astronomy. Among endangered Discovery projects are the CONTOUR mission to tour several comet nuclei, the Deep Impact asteroid probe and the

MESSENGER mission to explore Mercury. Also under threat are the Gamma-ray Large Area Space Telescope, US participation in Europe's Far Infrared and Submillimetre Telescope, and the Pluto-Kuiper Express mission.

Two Earth science projects would be lost: the Triana spacecraft to provide continuous pictures of Earth, and an Earth-viewing radar called LightSAR. Work to develop a follow-on to the Earth Observing System multi-instrument platforms would stop.

The cuts would hit academic scientists too: NASA estimates that a \$35 million reduction in its research budget would eliminate 600 grants.

Tony Reichhardt

Astronomers deflect scare-stories heading for media overkill

Boston

The International Astronomical Union (IAU) is ready to implement guidelines designed to ensure that predictions of asteroids heading for the Earth are vetted before they appear in newspapers.

The guidelines were written at a meeting in Torino, Italy, after last year's predictions that the asteroid 1997 XF11 might hit Earth in 2028. Astronomers were ridiculed after the calculations were revised. The meeting also agreed the Torino Impact Hazard Scale, to quantify risks.

"The guidelines are designed to ensure that accurate and verified information is presented to the public," says Richard Binzel, an astronomer at the Massachusetts Institute of Technology and chief architect of the hazard rating system.

The voluntary guidelines may not become official until next summer. They call for astronomers to make their data available to an IAU-assembled review panel if they discover an asteroid rated one or higher on the Torino scale, meaning an Earth impact cannot be ruled out.

The review team would check calculations within 72 hours. If it concludes there is a significant impact risk, its analysis will be made public on the IAU website.

The guidelines have been circulated to some 600 scientists worldwide, says David Morrison of the NASA Ames Research Center, president of the working group that devised them. "If somebody comes up with a prediction of a potential impact, they could use this service today," he says.

"Astronomers are free to go public without seeking this peer review," adds Hans Rickman of the Uppsala Astronomical Observatory in Sweden, the IAU's assistant general secretary, "but their statements would have less credibility than if they'd gone through the process and had their calculations checked."

Steve Nadis



France to renovate natural history museum

Paris

The French science ministry has vowed to end decades of neglect of the Natural History Museum in Paris, and intends later this month to announce a ten-year, FF2.6 billion (US \$420 million) renovation as part of its budget for 2000.

The government also stepped in last week to address the museum's much criticized overall strategy and administration. Instead of appointing a successor to palaeontologist Henry de Lumley, who has just ended his term of office as director, it appointed an emergency interim administrator, Jean-Claude Moreno, charged with preparing a major reform of the museum.

For the research aspects, Moreno — who admits to having little knowledge of science — will be aided by a “committee of scientific orientation” of top museum scientists, including foreign ones. “We need to learn from the experience of those elsewhere,” says Moreno.

The Paris museum's architecture and research collections are in decay, it suffers from bureaucracy, and it lacks a strategy to balance its core missions: research, informing the public, and the curation of its collections.

De Lumley has been credited with making substantial improvements. But decades of financial neglect have left the museum in need of an ambitious rescue programme.

The top priority is renovation and safety, says Vincent Courtillot, director of research at the science ministry. Reports by independent government panels have warned that much of the museum fails to meet safety requirements — staff are at risk from fire, electrocution, dangerous roofs and poorly stored chemicals (see *Nature* 385, 378; 1997).

The Paris research collections are rivalled only by those of the Smithsonian Institution in Washington and the British Museum of Natural History in London. They include 2 million fossils, 150 million insects, and 1.5 million vertebrates.

The Smithsonian Institution has transferred 20 million specimens to a purpose-built storage and research centre just outside Washington, but collections in Paris are often piled in boxes in corridors. The refurbishment programme should provide storage and research conditions to keep specimens free from insects, dust, and extremes of temperature and humidity.

Philippe Janvier, a vertebrate paleontologist at the museum, welcomes the promise of new money, but will “wait and see” if it appears. He says that several previous plans, including a much needed renovation of the palaeontology gallery, have ended up gathering dust with the specimens.

But Courtillot and Moreno insist that physical renovation must be accompanied by a modernization of the museum's activities.



Time for a change: the Natural History Museum in Paris is in urgent need of modernization.

Moreno is no stranger to the museum. He oversaw the FF500 million renovation of its Grand Galerie (see *Nature* 362, 280; 1993), completed in 1994, and chairs the state committee responsible for national cultural public works, which proposed the renovation plan.

The interim administrator will have broad powers and does not have to answer to the museum's board or scientific council. Trade unions representing museum staff, which are traditionally wary of government intervention, have welcomed the imposition of direct rule, as has De Lumley.

Under a century-old system, the director

and the museum's 26 professors have controlled its laboratories, public exhibition halls, zoos and research collections. But a new management structure seems likely to emerge. “Businessmen and not scientists” should run the museum's non-scientific activities, says Courtillot, arguing that past management has been “incompetent”.

Courtillot says the aim is to “confirm and accelerate” the core activities. External funding agencies will need to take scientists' curation work into account in evaluation, he says, given the importance of research collections to biodiversity and environmental research (see *Nature* 394, 115; 1998).

Courtillot adds that “it is an understatement to say that the research at the museum is not high quality”. Individual laboratories are likely to be regrouped as institutes addressing key topics.

The reform is likely to come as a relief to researchers. Administration of the museum has been a “veritable catastrophe” says Janvier, complaining of bureaucracy, long delays in processing grants, and antiquated management tools and methods.

Declan Butler

Busquin targets young people

London

The European Union (EU) needs to work harder to increase the popularity of science among the young, says the next leader of its research programmes.

Philippe Busquin, president of Belgium's Socialist Party, signalled his priorities for the EU's impending sixth Framework programme when responding to questions from the European Parliament, which is expected to confirm his appointment as EU research commissioner next week.

He will replace science and education commissioner Edith Cresson, who was at the centre of mismanagement allegations that forced the resignation of the commission in March. But Busquin will concentrate fully on the EU research portfolio. Detailed planning for the sixth Framework programme will begin early next year.

Busquin told the MEPs that Europe needs to attract young people into research to bring the proportion of scientists in the population up to US or Japanese levels.

He also said Europe should “exploit the relative advantage it possesses in areas such as aeronautics, but also pay attention to others with a high job-creation potential, such as biotechnology”.

Regarding the possible division of future Framework programmes into basic and applied research, he said there should be “no



Busquin: incoming EU commissioner.

dichotomy” between the two branches of research. Each area of the fifth Framework programme “contains an element of basic research”, he said.

Busquin said he would stage a debate on the future of EU nuclear fusion research following the loss of US support for the

International Thermonuclear Experimental Reactor project.

Busquin's suitability for the job was challenged on both academic and moral grounds. Spanish MEP Alejo Vidal-Quadras Roca said Busquin had no “significant scientific qualifications,” but Busquin has a first degree in physics from the Université Libre de Bruxelles, where he was an assistant lecturer for 15 years. He was also chairman of Belgium's Institut de Radioélément and energy minister for the state of Wallonia.

Asked about his involvement in past political scandals and illegal methods used to finance his political party, Busquin said he had never been accused of any illegal transactions or fraudulent behaviour and that the financing problems arose before he was elected president.

Keith Nuthall

Battle lines drawn between 'nanobacteria' researchers

Munich

A Finnish scientist has formally asked the University of Kuopio to investigate the work of one of its senior researchers, who, he says, is making misleading but widely publicized claims to have discovered a new form of life, known as nanobacteria.

Over the past few years, a group of Finnish scientists have expressed concern that Olavi Kajander has failed to produce the necessary biochemical evidence to prove that the particles he claims to be nanobacteria are in fact alive. Kajander says that he has shown this, and he has supporters of his own, including some prominent researchers at the US space agency NASA.

Kajander's critics object to his contention that not only do nanobacteria exist, but that they could have important medical implications, and could represent a model for primitive life on Earth. They charge that Kajander's promotion of his theories is unethical, because it has encouraged other researchers to develop the work before the fundamental issue of the particles' biological nature has been clarified.

Kajander first identified small bacterium-like particles in biological fluids, under particular culture conditions, ten years ago. He describes them as spherical, with diameters of between 50 and 500 nanometres. They are heavily coated with hydroxyapatite, which Kajander says renders them highly resistant to chemicals and heat.

They could thus, he says, be the Earth-bound equivalent of the fossilized structures observed in a meteorite assumed to have come from Mars, which some scientists regard as evidence of life there. Kajander says that their tough coats could have allowed the nanobacteria to survive the heat of entry into the Earth's atmosphere hidden within meteorites. He is now formally associated with NASA's Institute of Astrobiology, a virtual institute involving eleven research centres in the United States, collaborating with David McKay of the Johnson Space Center in Texas on Mars rock research.

Kajander's evidence of biological material in his particles is dismissed by his critics as the result of possible contamination, or of faults in experimental design or interpretation. Kajander denies this, and claims that their exotic properties mean that standard biological tests are irrelevant to nanobacteria.

Critics counter that it is not necessary to invoke exotic properties for phenomena that could be more simply explained. They say that the apparent replication of the particles, for example, could be explained by crystallization from the culture medium.

Jouni Issakainen, a mycologist at Turku University Central Hospital, says that "wide epidemiological conclusions should not be drawn until the possible microbial nature of the particles is independently and critically assessed".

In a letter published last week in the Finnish science magazine *Tiede 2000* (Science 2000), Issakainen alleges that Kajander selects electron micrographs and other morphological evidence to support his theory, and may even use technical tricks to generate structures that appear to resemble small bacteria. In a paper published last year in *Proceedings of the National Academy of Sciences* (PNAS 95, 8274–8279; 1998), Kajander claimed that he had identified DNA in nanobacteria. Issakainen says this cannot be substantiated because controls were not shown, and because Kajander increased the normal concentration of a DNA stain by an order of magnitude, as well as increasing the reaction time. Under these conditions, the stain can become nonspecific, says Issakainen.

Issakainen has written to the rector of the University of Kuopio requesting that the university investigate his suspicions that Kajander has "at least reported his observations carelessly, but possibly also had an intention to mislead".

Matti Saraste, a scientist at the European Molecular Biology Laboratory in Heidelberg, agrees that "caution should play a

major role until clear biochemical evidence that nanobacteria are living has been provided". Kajander has had years to prove his case, he says, "and the necessary tests are simple enough".

In a recently published article in the Finnish newspaper *Helsingin Sanomat*, he warns that Kajander's nanobacteria theory is developing into a "debate between two schools of thought, with proponents ... defining their opponents as unbelievers who are reluctant to accept new knowledge". A combination of "uncritical conduct of research, wishful thinking and a wish to gain publicity" could lead the public, and scientists themselves, to forget that the theory has — at least as yet — no basis, he warns.

The controversy intensified last year following publication of the PNAS paper, which implicated nanobacteria in the formation of kidney stones. The publication put Kajander's work firmly on the world stage.

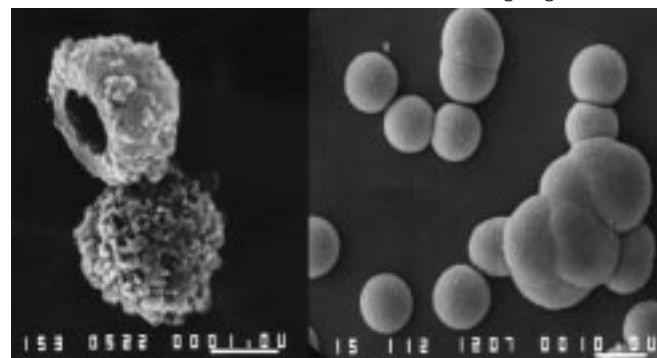
Kajander complains his critics are simply prejudiced against the idea of nanobacteria. They do not argue scientifically, but "malevolently," he says, and without care for the potential medical benefits of his work. "In fact, I don't care whether nanobacteria have genetic material or not — we have shown that they are automatically replicating particles that produce apatite, and that they are involved in disease. And I want to cure disease."

Kajander has supporters who are building on his work. "We think that Kajander has proved the nanobacteria are living," says McKay. He intends to work with Kajander to develop nanobacterium biomarkers for testing Martian rock samples returned from future NASA missions.

Kajander's announcement that he has identified purported nanobacterial particles in 90 per cent of kidney stones has excited interest in several US research groups. Tom Hjelle, an associate professor of pharmacology at the University of Illinois, says that his group has morphological evidence that the nanobacteria he sees in polycystic kidneys can grow and divide. "There is serious interest out there among physicians and commercial companies in the idea of nanobacteria," he says. "And in the next year or so we will see a lot of reports being published."

Despite the controversy, the medical and scientific sections of the Academy of Finland have continued to support Kajander's work. Reviewers of a FM706,000 (US\$120,000) three-year grant application for work on nanobacteria "combining microbiology, geology and astrobiology" recommended rejection, but the grant was approved after the publication of the PNAS paper, and funded with money from the academy's 'risk fund'. "Although we recognize that there are no solid elements of a scientific basis, the academy decided to take a risk with the work," a spokesperson says. **Alison Abbott**

O. KAJANDER



Life as we know it? Kajander believes that structures like these 'castles' with thick mineral shells (left; scale bar, 1 µm) and the larger, shell-less blobs (right; scale bar, 10 µm) are living nanobacteria.

Three Rs could make laboratories a better place for animals

Munich

Hundreds of scientists and animal welfare sympathizers last week signed a declaration endorsing the 'three Rs' principle. This advocates reducing the number of animals used in research, replacing animal experiments wherever possible and refining experiments to reduce suffering.

The declaration was circulated at the Third World Congress on Alternatives and Animal Use in the Life Sciences, held in Bologna, Italy, last week. More signatures will be sought from members of scientific societies.

The declaration is not controversial in most western countries: the principle was formulated 40 years ago and has been the basis of discussions between scientists and animal-welfare groups for years. But Michael Balls, head of the European Centre for the Validation of Alternative Methods, based at Ispra in northern Italy, says it will lend formal support to those seeking better treatment of animals in developing countries. Balls was one of the initiators of the declaration.

The total number of animals used has fallen dramatically during the past two decades, as cheaper *in vitro* methods have been developed to screen drugs and test toxicity. But the number used in basic research has not changed, possibly because of the growing usefulness of transgenic animals.

Alison Abbott

Universities and business fight over right to information

Washington

New guidelines proposed by the White House are unlikely to settle an increasingly rancorous dispute between the US research community and business interests over public access to research data. Both sides say the matter will probably end up in litigation unless Congress amends or erases the law that started the argument.

Researchers and their business opponents have both rejected revised guidelines issued by the White House Office of Management and Budget (OMB) on 18 August. The revisions have been in progress since late last year, when Congress approved an amendment to an appropriations bill by Senator Richard Shelby (Republican, Alabama) which made universities and other non-profit research institutes subject to the Freedom of Information Act (FOIA).

The amendment is supported by business interests, which believe it will enable them to challenge university research that the government may use to justify various regulations. It has been opposed by the universities, which find it unacceptable that they should be subject to FOIA. They want to keep control of their data and believe the amendment could lead to harassment of researchers.

OMB proposes that FOIA requests could only be made for research that underpins government regulations, and only where the regulations are likely to have an economic impact exceeding \$100 million. Laboratory

samples, preliminary analyses, plans for future research, peer reviews, personnel and medical files, trade secrets and intellectual property would not be accessible.

The Association of American Universities, which represents most of the top research universities, says the revision is too vague about who will pay compliance costs. The universities want to retain the right to decide what material can be sent to the agencies, and are concerned that intellectual property and the privacy of research subjects will not be adequately protected.

The US Chamber of Commerce, which has championed the Shelby amendment, believes the OMB guidelines have undermined the amendment's intent. "We have trouble with the \$100 million threshold," says Louis Renjel, a member of the chamber's regulatory affairs staff. "That narrows it down to only one per cent of all regulations the government issues." He says the chamber will insist on the amendment covering all government research grants, in all fields relating to all federal rules and regulations.

The conflict might have been avoided if OMB had kept White House science adviser Neal Lane informed of its negotiations with Shelby late last year. Milton Goldberg, director of the Council of Government Relations, a group representing universities, says: "It's true that OMB dropped the ball here. They probably didn't appreciate the amendment's significance in its early stages." **Will Lepkowski**

Policy reversal puts science on top in New Zealand

Sydney

In a late policy change three months before a general election, New Zealand's governing National Party has decided to promote science and innovation as a top priority.

Prime minister Jenny Shipley has launched a five-step package aimed at reversing New Zealand's comparatively weak support for university and industrial research.

Though extra funding is small, the package is a substantial shift from the austere research policies that have characterized the past 14 years under successive governments (*Nature* **398**, 450; 1999).

In recent years, university support has fallen on a per-student basis, while fees have risen. The government jettisoned a target set in 1996 to boost its research expenditure from 0.52 to 0.8 per cent of gross domestic product by 2010, and ignored repeated calls for industrial research incentives.



Shipley: reversing years of austerity.

Under the new package, task forces would be set up to examine higher education and tax incentives for industrial research. Eighty scholarships for doctoral students and 26 new post-doctoral fellowships are to be created, with extra funding of NZ\$47 million (US\$24.5 million) over four years.

The National Party currently trails the opposition Labour Party in opinion polls. Labour, led by Helen Clark, has yet to release its election platform, but has said it will include more support for basic research and tax incentives for industrial research. Shadow science minister Mark Peck proposes a new, independent council of scientists to advise the prime minister.

Industry reactions to the package were broadly supportive, while those from academic and science leaders were more mixed.

John Hood, vice-chancellor of Auckland University — New Zealand's largest — welcomed the package as acknowledging "serious weaknesses in the present policy framework". But Daryl Le Grew, vice-chancellor of Canterbury University, dismissed it as "fragmented and something of a knee-jerk reaction".



Clark: tax breaks for research?

Andy West, chair of the association of the government-run Crown Research Institutes, said that the change was "most welcome and long overdue" but called for total research expenditure to be doubled or trebled.

Peter Pockley

GM backlash leaves US farmers wondering how to sell their crops

American farmers have warmly embraced biotechnology. But resistance abroad and regulation at home are threatening to turn the affair sour.

As the consumer movement against genetically modified (GM) foods spreads across Europe, Japan and elsewhere, maize (corn) and soybean farmers preparing for harvest in the United States face a shrinking export market, together with growing demands from food processors that they separate GM from conventional grains.

While American farmers generally like the convenience of the built-in pesticides featured by GM crops, they are fearful that their harvest will fail to find a buyer.

On 1 September, food-processing giant Archer Daniels Midland asked suppliers to segregate fields, grain bins and storage elevators. Consolidated Grain and Barge, another major processor, said it would pay a premium for unmodified crops. And several US food companies, including Gerber, H. J. Heinz and Iams, a pet-food maker, have started to reject GM varieties.

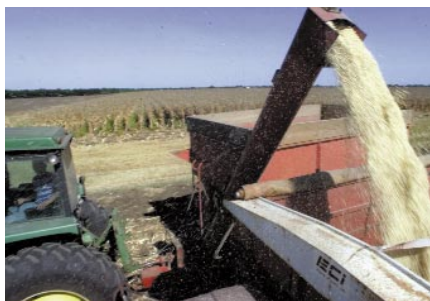
Resistance spreads

The American Corn Growers Association (ACGA), a progressive farmers' group, has advised its members to stop planting maize modified to produce pesticides. Separately, the Organic Trade Association is debating whether to fight the regulatory renewal of crops engineered to contain insect toxins, and has produced guidelines to protect organics from 'genetic trespass'. Some maize and soybean farmers who planted GM seed are even talking about class-action lawsuits against seed and chemical companies for misrepresenting their products as benign.

Gary Goldberg, chief executive of the ACGA, says that Archer Daniels Midland's demand is a wake-up call. "GM organisms have become the albatross around the neck of farmers," he says.

The corn harvest has begun in Texas and will reach the Midwest in mid-September. GM maize accounts for more than one-third of this year's grain, and few have tried to separate it out. But Japan has now announced a labelling programme, a major Mexican tortilla maker has banned GM maize, and the European Union has barred several of the most widely used GM varieties (see *Nature* 398, 736; 1999).

One-fifth of the maize harvest was sold overseas last year; Japan, the biggest customer, bought about one-third of the



Lost in a maize? Farmers face a hard time keeping GM and non-GM grains apart.

exported crop. Korea and Mexico followed, with Europe buying most of the maize gluten for animal feed.

Now that there is a market for unmodified grain, an important farmers' concern is 'contamination' of such grain by GM crops. Modified and unmodified seeds can mix in harvesting equipment, storage elevators and processing machinery.

For maize and canola, cross-fertilization by wind-borne pollen is a risk. Growers say they are starved for data on the need for buffer zones, and wonder aloud who would be liable if GM pollen transformed unmodified crops. Organic farmers, who feel especially threatened, say that even the identity of seed stock is uncertain.

Growers may not be able to comply with requests for grain segregation, warns Susan Keith, public-policy director for the National Corn Growers Association, the other main maize-farmers group, which is more supportive of GM crops than the ACGA. Keith says food processors are being overly cautious, adding that the demand for identifiable unmodified grain may make it hard for US maize farmers to sell this year's crop.

Producer groups worry about the cost of tests to ensure that crops are not contaminated. The American Soybean Association has released a statement saying that consumers and processors ought to pay a premium to cover the extra expense of certifying unmodified crops. (GM plants make up about half of this year's soybean crop.) Organic growers wonder if such testing is even possible, especially in processed foods such as soybean oil or lecithin, as transgenic crops become ubiquitous in the food supply.

"As more [organic] processors are testing the ingredients that they're receiving, we're

finding other cases," says Katherine DiMatteo, executive director of the Organic Trade Association in Greenfield, Massachusetts.

Organic farmers are afraid that GM crops will rob them of some key management tools. The spread of plants containing a toxin from the bacteria *Bacillus thuringiensis*, for example, could create resistance to Bt sprays amongst the caterpillars and moths that ruin maize, cotton and potatoes.

Bt or not Bt?

The Environmental Protection Agency (EPA) is concluding a series of workshops on managing fields to protect against resistance to Bt toxins in maize and cotton. In its position paper, the agency praises GM crops for helping to boost production and cut chemical use. The EPA cites figures from the biotechnology company Monsanto that cotton insecticide use dropped by 3.6 million litres per year after Bt cotton entered the market. Farmers use about 300,000 kg less of insecticide because of GM maize, the agency says. In addition, herbicide-resistant varieties have improved erosion control by enabling farmers to avoid tilling.

But the Organic Trade Association may fight the renewal of these plants' status as approved pesticides, says DiMatteo. Besides potentially eliminating a safe insecticide, she says, these crops pose unknown risks to the environment: "It's trading one bad solution for another". Expanding organic acreage would be a better idea, she says, especially because organic maize is easy to grow.

The EPA is evaluating the emerging data on harm to non-target insects such as the monarch butterfly, says Steve Johnson, EPA associate deputy assistant administrator for the Office of Prevention, Pesticides and Toxic Substances. "We really believe the science is raising some important concerns," he says.

The EPA may expand requirements for insect refuges and untreated buffer zones. In addition, companies requesting registration renewal may be asked to provide data on ecological questions such as the effect of Bt pollen on monarch habitat, the dose-response relationship for different butterfly life stages, and the relationship between monarch colonization of milkweed and the distance to a maize field. "To the extent that we need to be more aggressive as an agency, we will be," Johnson promises. **Sally Lehrman**

More researchers sought in German medical schools

Munich At least a quarter of all positions at German university medical departments should be held by full-time research scientists, says a report on the state of clinical research. It also recommends improving career opportunities for basic researchers at Germany's 36 university hospitals.

The report was prepared by the Deutsche Forschungsgemeinschaft (DFG), the university grant-giving agency. It finds that the results of clinical research in Germany are "not satisfactory" because research is poorly established in medical schools.

There is hardly any laboratory space available in some hospitals, according to the report. "Research is widely regarded as a leisure time amusement," it states. "Really concentrating on doing good research often means a career risk for clinicians."

No evidence yet for water on the Moon

Washington NASA's Lunar Prospector spacecraft has failed to find evidence of water on the Moon, since its intentional crash there this summer. But researchers are still looking at

data from the experiment, which was not expected to settle the controversial question.

All indications are that the Lunar Prospector crashed into a shaded south-pole crater as planned on 31 July. But none of the hundreds of professional or amateur ground-based telescopes watching have reported seeing signs of water vapour or its by-product, the hydroxyl (OH) molecule, splashing up from the impact. A University of Texas team led by David Goldstein is doing final checks of spectra from the Hubble Space Telescope, the McDonald Observatory in Texas and Hawaii's Keck I telescope. They hope to have a verdict in a few weeks.

Chemical trio set up joint genome company

Tokyo Mitsubishi Chemical, Kyowa Hakko Kogyo and Yoshitomi Pharmaceuticals announced last week that they have set up GenCom, a joint genomics venture focusing on the analysis of human gene functions.

This is the first venture company in Japan to carry out research on functional genomics. It will perform protein analyses to identify genes useful for medical research and develop transgenic animals that could be used as models for drug development.

GenCom plans to conduct research and development for private companies and

public research institutes, using genetic and protein engineering technology developed by Mitsubishi Chemical, which has a 67 per cent stake in the company.

The company plans to seek funding from Japan Key Technology Centre, an organization set up by the government in 1985 to support research in biotechnology and telecommunications.

Japanese ministry plans to merge institutes

Tokyo Japan's Ministry of International Trade and Industry (MITI) is planning to merge all 15 research institutes operating under its Agency of Industrial Science and Technology (AIST) into a single institute with greater administrative independence.

The change is part of the government's wider plan to improve administrative efficiency through a large-scale reorganization of its ministries and agencies (*Nature* 389, 897; 1997).

National research institutes and universities have been targeted by the government plan, which is based on the UK Next Steps reform strategy. But researchers have voiced concern, saying that proposed performance-related targets, especially those related to costs, could jeopardize basic research.

France refuses to lift ban on British beef

Paris France has decided to opt out of the decision taken by the European Union (EU) this summer to lift the export ban on British beef.

Instead, the government has asked the French food-safety agency to give an opinion on the safety of British beef. A team of scientific experts led by Dominique Dormont, a prion expert who also heads the French government's spongiform encephalopathy advisory committee, is not expected to make recommendations before the end of this month.

The decision to maintain a *de facto* embargo is protected under a law passed last year to ensure food safety, but it could have political ramifications with the EU. Germany may also defy the EU decision and maintain its ban on the import of British beef.

Parliament quizzes biotech body over code of conduct

London The BioIndustry Association (BIA) last week faced questions in a UK House of Commons Select Committee over its draft code of practice. This was introduced partly in response to events at British Biotech, whose head of research was sacked in 1997 after casting doubts on the effectiveness of the

company's two main drugs, causing a drop in share prices that hit the UK industry.

The BIA's code, if it is approved at its general meeting later this year, will apply to its 200 member companies. Although the BIA and the London Stock Exchange say the existing regulatory regime is satisfactory for biotechnology companies, both are eager to push a code of practice.

The code covers issues such as the appointment of non-executive directors, the timely release of information, and the seeking of advice from outside experts. Transgressors would face censure or expulsion from the BIA.

Diabetes drug inquiry has second target

San Diego Federal investigators are reportedly opening an inquiry into a potential conflict of interest by a second researcher involved in a National Institutes of Health (NIH) trial of the diabetes drug Rezulin.

An NIH report published last week said that the agency's Office of Inspector General will examine the role of Jerrold Olefsky, a physician at the University of California at San Diego (UCSD), in an NIH diabetes study of Rezulin. Olefsky, who reportedly holds three patents associated with the drug's use for diabetes, is on leave from UCSD to

work with a biotech firm, and could not be reached for comment.

The Inspector General earlier this year began a conflict investigation of the NIH's diabetes division director, Richard Eastman, after questions were raised about his acceptance of more than \$75,000 in consulting fees from Rezulin's maker, Warner-Lambert. Since Rezulin was approved for diabetes use in 1997, the firm has repeatedly increased drug safety warnings after users suffered liver damage.

China launches its biggest science prize

Beijing China has approved the establishment of its largest ever scientific award, the State Supreme Science and Technology Prize.

The prize will be up to 5 million RMB (about US\$620,000). However, the winner will pocket only 500,000 RMB, with the rest being used to support research in a field of his or her choice.

Up to two of the awards will be conferred each year by President Jiang Zemin to Chinese citizens who have made a key breakthrough, have made an outstanding contribution to the advancement of science and technology, or have brought about social and economic benefit through the application of their work.

A science-oriented search engine could solve web problems ...

Sir — Lawrence and Giles eloquently describe the problems with the World-Wide Web — the small percentage of total pages indexed by search engines and their bias towards 'popular' pages (*Nature* **400**, 107–109; 1999). This suggests that a large proportion of the scientific information on the web, from home pages to preprints and sequences, may never be discovered. But the authors also point to a way forward.

Only six per cent of web servers have scientific or educational content — a much more manageable amount to index. Metadata (information about information) is the key to better searching, yet only 0.3 per cent of servers use the Dublin Core standard for metadata: <http://purl.org/dc/>. We need a science-oriented search engine, together with a set of scientific metadata, to help us trawl the oceans of information.

I think that a hybrid indexing scheme, halfway between the formidable task of a human-generated Internet catalogue like Yahoo! and the huge computer-generated indexes like AltaVista, is the way forward. Submission of an institution's home page would, after manual verification to exclude inappropriate sites, lead to the computerized indexing of the rest of the site.

The next step is for the scientific community to discuss suitable extensions to the Dublin Core metadata to describe the rich variety of scientific information available on the web. *Nature's* debates — at <http://helix.nature.com/debates> — would seem to be the ideal forum.

Mike Gardner

School of Biomedical Sciences, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK

... just try to be specific ...

Sir — It is disturbing that no single search engine indexes more than a meagre 16 per cent of the web, down from about 33 per cent 18 months ago (*Nature* **400**, 107–109; 1999). Users' ability to look at documents has remained static, so people still only look at the first few tens of search results.

Most mainstream search engines rely on relevance-score algorithms to rank matches. Such algorithms are prone to manipulation by content providers, who can rig their content to yield a spuriously high relevance score. But transparent algorithms are necessary for users to understand how to get the most from search engines. The end result is a long list of hits of doubtful relevance, quality and completeness.

This makes topic-specific search engines — which aim to cover the majority of content within a specific topic, rather than a small fraction of every topic — all the more appealing. Such an approach also allows content to be reviewed and structured so that users are presented with an intelligently categorized, hierarchical list of matches, rather than a linear one. This allows rapid identification ('drilling down') of the most relevant matches.

One example that addresses these issues is OrthoSearch (www.orthosearch.com), covering orthopaedics and related topics. OrthoSearch uses the Internet Society of Orthopaedic Surgery's web-links policy to determine which sites are relevant, avoiding many shortcomings of the most popular search engines.

A. L. S. Chiu*, E. Sherry†, X. Phung§

**Department of Anatomy and Histology,*

†Department of Orthopaedic Surgery,

§Westmead Hospital, The University of Sydney, New South Wales 2750, Australia

... using peer review as a guide to quality

Sir — A statement often attributed to the late science fiction editor John W. Campbell says: "90 per cent of science fiction, indeed 90 per cent of anything, is garbage". With this in mind, the recent report that Internet search engines index only an average of 7–16 per cent of the web becomes much less alarming (*Nature* **400**, 107–109; 1999).

Most *Nature* readers will agree that Campbell's law is eminently applicable to the Internet. The pressing concern is not what percentage of the web a search engine covers, but how much of the web is worthy of coverage and how to identify that fraction. The scientific community has long had a mechanism to categorize information worthy of attention: peer-reviewed publication. So, if one needs useful information on, say, nerve growth factor, PubMed is likely to be a more useful website than the Yahoo! search engine. One hopes that the architects of the 'E-Biomed/E-Biosci' initiative bear this in mind.

Mike Fainzilber

Molecular Neurobiology Group, Department of Biological Chemistry, Ullman Building, Weizmann Institute of Science, 76100 Rehovot, Israel

Money, money, money

Sir — Vasiliki Plerou *et al.* suggest that the similarities between the growth dynamics of university research and of businesses may be due to the similarity of peer review and government direction to market forces, that is, consumer evaluation and product regulation (*Nature* **400**, 433–437; 1999).

I can think of another possible cause: that the goal of universities has now become similar to the goal of businesses — to make money.

Barbara-Ann G. Lewis

Department of Civil Engineering, Northwestern University, Evanston, Illinois 60202, USA

Will Explore set the next century's standards?

Sir — Heather McCabe's news article "UK centre drops science for sensation" (*Nature* **400**, 804; 1999) gives a false impression of Explore at Bristol, and of the relationship between Explore and the Exploratory.

Explore is part of a £97 million millennium project in the centre of Bristol. In seeking to widen the appeal of science, nature and the arts, two new centres (Explore at Bristol and Wildscreen at Bristol) and a public art programme in the new squares and walkways are being created. Explore at Bristol focuses on people, their brains and how these affect the way they see the world, their dreams for technology, their place in evolution and their impact on the planet. The centre is covering new subject areas and widening the range of media used. But it is most certainly building on its inheritance from the Exploratory, and a 'hands-on' approach is at its heart.

Many of the Exploratory exhibits will be used, with one-third of Explore at Bristol exhibits being the same as Exploratory exhibits; more than two-thirds of all the exhibits in Explore will be hands-on. Professor Richard Gregory was the first to introduce hands-on science to the United Kingdom in Bristol about 16 years ago. We believe we are building on this concept and extending it to an even broader public.

Hands-on science is most effective for eighteenth- and nineteenth-century science and fundamental phenomena: light, electricity, magnetism, and so on. For many newer areas such as life and biomedical sciences, or different aspects such as the social implications of science, different techniques are needed. We want the best method for communicating messages and encouraging people to explore the world around them. Exhibits will be grouped in themes and the hands-on approach reinforced by other types of experience and information.

Two nominees of the Exploratory Trust have been on the board of Explore at Bristol since 1995; Gregory, who founded the Exploratory, is chairman of the science advisory panel for Explore at Bristol and an honorary life vice-president. Gregory himself said of Explore recently, on BBC Radio Bristol's *John Turner Programme*,

"They are actually using a lot of our exhibits and no doubt will develop and improve them because they've got more facilities. From the point of view of Bristol ... I think actually there may be a gain."

Just as the Exploratory set new standards when it opened, so Explore at Bristol is striving to set new standards for the next century. We hope readers will come and see for themselves when we open — on time, and on budget — next spring.

Gillian Thomas

Chief Executive, at-Bristol, Deanery Road, Harbourside, Bristol BS1 5DB, UK

McCabe replies — My article correctly reports the relationship between Explore and the Exploratory. But, as chief executive of at-Bristol, Gillian Thomas naturally does not agree with the criticisms I reported of Explore's approach to science.

Although Thomas says that Explore is carrying on the tradition of the Exploratory, many scientists involved in planning the new centre believe that the scientific content of Explore is thin. Thomas and her colleagues pointed out to me examples such as brains you can touch, crickets you can look at through a magnifying glass, and a virtual-reality sperm ride. Although these are hands-on in a literal sense, they do not oblige visitors to run through a mini-experiment to observe a scientific principle.

The scientists I spoke to embrace the idea of having a centre with a wider appeal and a budget to build more modern exhibits, as Thomas stresses that Explore is doing. Yet they say that, in the rush to open in a timely fashion for the millennium, Explore is creating expensive exhibits that favour special effects over scientific substance.

Spanish recruitment openly favours insiders

Sir — Rigidity and cronyism characterize hiring practices for academic positions in Spain (*Nature* **396**, 709; 1998). This happens both in universities, as denounced in your pages, and, to a lesser extent, in the Spanish Research Council (CSIC), the country's largest research body. This is demonstrated by two worrying developments during the past few months.

First, in order to 'stabilize' the situation of university lecturers on short-term and irregular contracts, the government and university vice-chancellors have proposed promoting 10,000 of them to permanent lecturer positions. This highly irregular upgrade is, however, closed to equally qualified postdocs in non-university research institutes (such as CSIC) or abroad. It would result in a freeze on university hiring for the foreseeable future, leaving non-university centres and foreign

countries as the only outlets for researchers seeking tenure-track academic jobs.

Second, CSIC has announced 90 new research positions, for which candidates are assessed on a score of up to 20 (*Nature* **399**, 400; 1999). Ten points have to be earned on merit (including publications and experience) to reach the final selection process. But five points are given as a 'prize' to people who have worked in CSIC — putting other candidates at a clear disadvantage as they can only obtain a maximum of 15 points.

Job openings at CSIC and universities are not widely advertised, and bureaucratic requirements make them almost unattainable by outside candidates (*Nature* **400**, 203; 1999). For example, the 90 CSIC posts were only advertised in the Spanish Official Bulletin — an obscure government publication that is not widely available — and on the CSIC website. Applications typically have to be in within two weeks. Foreign qualifications require government 'validation', a process that can drag on for up to a year, discouraging candidates applying from outside Spain.

The solution to this cronyism is readily at hand. CSIC, universities and the Spanish government should simply follow the hiring policy of Spain's National Centre for Cancer Research, which is also common practice in Britain and the United States: job advertisements should appear in scientific journals, with plenty of time to apply, in order to attract the best candidates. The government should also remove bureaucratic obstacles that prevent outside scientists being hired.

Other problems exist in Spanish science, including 0.8 per cent of GNP dedicated to research (against a European average of 2.1 per cent), and a lack of research facilities and positions. But before solving those it is necessary to eradicate cronyism. This is one of the goals of the Association for the Advancement of Science and Technology in Spain (AACTE: <http://www.aacte.net>). It is the only way to attract good researchers, provide a healthy and flexible science base and high-quality education, and have a commitment to excellence in science.

Javier Escartin

IIA-CSIC, Martí Franqués s/n, 08028 Barcelona, Spain and 23 others

New opportunities for expert witnesses in court

Sir — Many scientists are required to give expert witness in civil litigation. Recent, sweeping reforms to English civil justice have radically changed the way scientists give their evidence. The agenda and rules of the courtroom have changed, and this may

provide new opportunities for scientific evidence to influence legal disputes. In particular the introduction of a single court expert, to replace the cross-examining of different parties' experts, will open new areas for scientists to use their expertise.

Civil law provides the basic structure within which commerce and industry operate, and safeguards the rights of individuals. In 1994, Lord Woolf was appointed to review the civil courts in England and Wales¹, resulting in the new Civil Procedure Rules (CPR)².

In English law, witnesses do not usually testify to anything but facts. However, the courts recognized the need for special witnesses providing evidence on both fact and opinion. The duties of expert witnesses evolved and were reinforced by case law³.

Although, in principle, the expert is independent of the instructing party, the system had flaws. These have been addressed in the Woolf review by introduction of new CPR, practice directions and forms. The new CPR came into force on 26 April 1999, and apply to all civil courts in England and Wales.

One key aspect of the changes is the duty of the expert to help the court, overriding any obligation to the person who instructs, or pays, the expert (CPR Part 35.3). The expert must include verification of the contents of any report with a statement of truth (CPR Part 35.10).

The court has a duty — not merely the power — to restrict expert evidence (CPR Part 35.1), and there is now a general requirement for expert evidence to be given in writing, removing many of the criticisms of the system resulting from adversarial cross-examination. Oral evidence and the attendance of experts at hearings will be restricted (CPR Part 35.5).

The changes to the civil justice system are as much cultural as procedural. All parties must be ready to be proactive, and must see this as the beginning, not the end, of the process of change.

The debate on the efficacy of expert witnesses appointed by the parties in dispute has raged elsewhere, notably in the United States in the late 1980s (*Nature* **378**, 754; 1995). Litigation should be the last, not the first, resort in attempts to settle a dispute, and this principle is central to the Woolf reforms. Experts now have greater scope to both affect and effect settlement.

Peter Fenn*, **Christine Jinks***, **Michael O'Shea†**

*Department of Building Engineering, UMIST, PO Box 88, Manchester M60 1QD, UK

†Masons Solicitors and Privy Council Agents, 100 Barbirolli Square, Manchester M2 3SS, UK

1. Lord Woolf *Access to Justice* (Woolf enquiry team, Room 438, Southside, 105 Victoria St, London SW1E 6QT, UK; 1995).
2. *Civil Procedure Rules* (The Stationery Office, London, 1999).
3. *National Justice Compania Naviera S. A. v Prudential Assurance Company Limited* (The Ikarian Reefer) CILL 838 (1993).

Gatecrashing the nuclear club

India's 1998 nuclear-weapons tests heralded a second nuclear age.

Dr Strangelove, I Presume

by Michael Foot

Gollancz: 1999. 231 pp. £16.99 (hbk),

£7.99 (pbk)

The Making of the Indian Atomic Bomb: Science, Secrecy and the Postcolonial State

by Itty Abraham

Zed Books: 1998. 174 pp. £13.95, \$19.95 (pbk)

Brahma Chellaney

India's nuclear ambitions have helped to mould international non-proliferation arrangements and have spawned many books. The 1974 atomic detonation in India, which its politicians described as a "peaceful nuclear explosion", triggered a series of major international developments. It led to the secret formation of the London Club of nuclear suppliers, the reshaping of the international non-proliferation regime, and the inclusion of dual-use items (those with both civilian and military applications) in Western technology-export controls.

It also had a major impact on US policy, leading to significant institutional reforms in export policy, the enactment of the 1978 Nuclear Non-Proliferation Act, the attachment of non-proliferation conditions to foreign aid, and the emergence of sanctions in response to proliferation.

After straddling the nuclear fence for almost a quarter of a century, India finally gatecrashed the nuclear club in 1998 by conducting a series of nuclear-weapons tests. The second nuclear age ushered in by India's action — part of the dramatic global changes taking place since the fall of the Berlin Wall — will complicate existing arrangements, including the Nuclear Non-Proliferation Treaty, which recognizes the right of only five countries to wield the ultimate weapons of mass destruction.

The books by Michael Foot and Itty Abraham are among a number of publications to come out after India armed itself so overtly. The contrast between the two is striking. Foot, Britain's former Labour Party leader, is sympathetic towards India and its security concerns, although he regrets its tests; Abraham, an Indian based in New York, comes out as a critic of the Indian nuclear programme. Foot writes from first-hand knowledge gained from interaction with leaders who shaped history; Abraham relies largely on secondary sources. Abraham employs the multi-disciplinary tools of sociology, comparative politics and international relations to present his scholastic and intricate arguments, while Foot provides a simple, straight-

forward political analysis.

Both authors are crusaders, but of a different kind. Foot's life-long disarmament advocacy runs through his book, although it does not unduly affect his historical narration. He wants a global "programme for the dismantling of the nuclear arsenals properly decreed and with a timetable", but recognizes that this has to be a step-by-step process.

Abraham's anti-nuclear views, however, get the better of his analysis. His central thesis is that India's nuclear ambitions are linked not to national security but to the "postcolonial state's project of modernity", and that they have made the country less, not more, secure. Nuclear scientists are described as the "high priests" of postcolonial India and he cites prominent anti-bomb Indian analysts with approbation. His book will be welcomed by the Indian anti-bomb lobby, which functions on the fringes of the national debate, and by those elsewhere who argue that India does not need nuclear weapons. But his commitment to one side of the debate weakens the force of his arguments.

The two authors provide contrasting perspectives on secrecy. Abraham accuses India of writing secrecy into the fabric of its nuclear programme and not giving scholars "full and unfettered access" to official nuclear records; he does not mention that no

nuclear-armed democracy grants such access. While focusing on secrecy, he also ignores the fact that India is the only democracy that, before adopting a nuclear military posture, debated for years whether to go nuclear. The other nuclear democracies (the United States, Britain, France and Israel) went down the nuclear path quietly, even stealthily, without any real public debate at home. Foot, however, writes about the "special brand of secrecy" which democracies such as the United States and Britain have applied in the nuclear area. He mentions the secrecy in the British bomb project, starting with 1945 when "the decision to make the bomb was taken by Prime Minister Attlee and a few of his closest colleagues without any reference to the House of Commons".

Abraham and Foot also provide dissimilar views on India's 1974 blast. Abraham says the detonation — "meant first to mark the rise of a new ruling clique within the Atomic Energy Commission and, second, to buttress India's claims to a renewed power and purpose" — worsened New Delhi's relations with its neighbours and changed its international status for the worse. Having created an unstable regional environment through that explosion, India 24 years later went overtly nuclear to "reinforce that instability", according to the author. Foot, in contrast, says, "The stated aim of the [1974] project was to harness atomic energy for peaceful purposes; and that claim was no deception". Foot assails "the hypocrisy of the nuclear nations" in claiming that nuclear bombs are just for some nations and that they "become explosive and dangerous in the hands of the brown skins".

Abraham is less cautious in drawing conclusions. For example, he declares emphatically, without citing evidence, that when India conducted its 1974 test, "nuclear weapons were no longer the single, universally accepted and valorised currency of international power". Similarly, India is "out of time" now, as it has "demanded its right to become a nuclear power just when the atomic age has come to an end, and thus remains an outsider, a spoiler".

One wishes the nuclear age had truly come to an end. Since India and Pakistan went overtly nuclear, the Czech Republic, Hungary and Poland have come under NATO's nuclear umbrella; Russia has placed tactical (short-range) nuclear arms at the centre of its defence strategy; China has tested an advanced long-range nuclear missile, and the United States has formally deployed a new Earth-penetrating nuclear warhead.



Hands up those who think that India did the right thing.

Today, no major economy is without the protection of an independent nuclear arsenal or a nuclear umbrella; and disarmament seems a dream, like nirvana. ■

Brahma Chellaney is at the Centre for Policy Research, Dharma Marg, Chanakyapuri, New Delhi 110021, India.

Counting on our brains

The Mathematical Brain

by Brian Butterworth

Macmillan: 1999. 480 pp. £20

Stanislas Dehaene

"One, two three, four. My mathematics finishes here." Those are the words of Signora Gaddi, an alert, 59-year-old Italian woman whose puzzling impairment has helped neuroscientists understand how the brain does arithmetic. Signora Gaddi suffered a stroke that damaged the left parietal lobe of her brain. Since then, she has become largely hopeless with arithmetic. She cannot read, write, compare or calculate with any numbers other than one, two, three and four. Even with numbers below four, she is definitely not performing normally. For instance, when shown two wooden blocks, she has to laboriously count on her fingers in order to establish their numerosity. Because Signora Gaddi performs normally on many other tests that do not involve numbers, her affliction can be described as a selective loss of arithmetic.

The detailed study of Signora Gaddi is just one of many fascinating pieces of evidence gathered by Brian Butterworth in his effort to illuminate the relations between brain, mind and mathematics in his book *The Mathematical Brain*. The title itself is something of a misnomer, for one would search this volume in vain for investigations of the cognitive bases of higher mathematics, or even of simple geometry, algebra or topology. The book focuses on a single mathematical object, but one that is rightly seen by Butterworth as a fundamental cornerstone of the mathematical edifice: the concept of number.

Butterworth's central hypothesis is that our brain is "born to count". Our genes contain instructions that specify how to build a number module, a set of neural circuits specialized for processing numbers. Those circuits, which are associated in part with the left inferior parietal lobe, make us sensitive to numerosities in our environment and allow us to understand and to manipulate numbers mentally. Loss of those circuits, as in Signora Gaddi's case, results in a selective inability to grasp the meaning of numbers. The number module is not unique to

humans: behavioural experiments reveal that many animals can also attend to numerosity. What makes the human numerical ability unique, however, is that it can be extended through the invention and spreading of cultural tools, such as number symbols and arithmetic algorithms.

In recent years, the cognitive neuroscience of numeracy, or 'numerical cognition', has emerged as an important area where the interaction between brain architecture and human culture can be studied empirically. The hypothesis of a modular architecture underlying number processing has been fruitful in many areas of research, from developmental psychology to brain imaging, animal behaviour or behavioural genetics. Several previous reviews of these findings are available, some aimed at specialists (for example, *The Nature and Origins of Mathematical Skills* by J. I. D. Campbell; Elsevier, 1992), others at a wider audience (for example, *The Number Sense* by S. Dehaene; Oxford University Press, 1997). *The Mathematical Brain* falls into the second category: it is a skilful overview of the area for the non-specialist, with remarkable depth and breadth in many cases, but with occasional oversights that may frustrate the expert.

Butterworth's review of prehistory is particularly original and commendable. He convincingly pulls together little-known evidence from cave-paintings and bone-carvings to suggest that the dawn of arithmetic in stone-age populations dates back at least as far as 30,000 years. More puzzling, however, is the almost complete omission of brain-imaging evidence in the discussion of the neural bases of the number module. Although the modern tools of positron emission tomography, functional magnetic resonance imaging and electro- and magneto-encephalography have been applied only recently to mathematical cognition, a review of the available evidence would have been welcome, especially since it confirms the presence of numerical circuits in a localized brain region: the left inferior parietal region.

Specialists will be delighted, however, by Girelli and Butterworth's latest evidence on developmental dyscalculia, some of which is published here for the first time. If there is a genetic plan for a number module, then one might expect to find an occasional child who is born without it, either due to a genetic defect or to pre- or perinatal cerebral damage. Butterworth claims to have identified one such patient, Charles, who is "born blind to numerosities". Although Charles is now a very bright adult, with a university degree in psychology, he has experienced profound, lifelong difficulties in mathematics, to the point of still having to count on his fingers in order to solve single-digit addition problems.

Chronometric tests reveal at least two major impairments. First, Charles cannot "subitize": he cannot decide how many items are presented on a computer screen, even if there are only two or three, unless he painstakingly counts them one by one. Second, he has an abnormal intuition of number size, which is reflected in an inverse distance effect in a number-comparison task: whereas we normally take less time to decide which of two numbers is larger as the distance between them gets larger, Charles takes more time for more distant numbers, presumably because he is using a very indirect counting strategy.

Charles has not been subjected to brain imaging, but another case of developmental dyscalculia, recently scanned with the novel technology of magnetic resonance spectroscopy, shows a small, isolated area of damage exactly where number circuits are postulated to lie — the left inferior parietal cortex.

The finding that early focal brain damage can have such a permanent and restricted effect on mathematical competence is perhaps the best evidence to date in favour of the number-module hypothesis. Such evidence imposes strong limits on brain plasticity and clearly speaks against purely constructivist theories that view mathematical competence as the result of a general learning device.

In the end, I suspect that Butterworth's hypothesis of a direct link between genes, number circuits and higher mathematical competence may be too simple. Still, the cogent arguments of *The Mathematical Brain* should be required reading for anyone interested in the modularity of higher cognitive functions. ■

Stanislas Dehaene is at Unité INSERM 334, Service Hospitalier Frédéric Joliot, 4 Place du Général Leclerc, 91401 Orsay cedex, France.

Biochemistry: a biography

Proteins, Enzymes, Genes: The Interplay of Chemistry and Biology

by Joseph S. Fruton

Yale University Press: 1999. \$45, £30

Charles Tanford and
Jacqueline Reynolds

F. Gowland Hopkins, Cambridge University's first professor of biochemistry and recipient of a Nobel prize for his work on vitamins, said in a lecture delivered in 1927: "Biology and chemistry, though in their infancy both foster-children of medicine and passing their childhood in company, have long occupied domains which, though never really far apart, have sometimes

seemed to be so. But their proper domains, while extending in all directions, have now definitely met at a frontier which is surely a region of supreme importance for both." (From *Hopkins and Biochemistry*, edited by J. Needham and E. Baldwin; Heffer, 1949.) That new frontier, of course, is now called biochemistry.

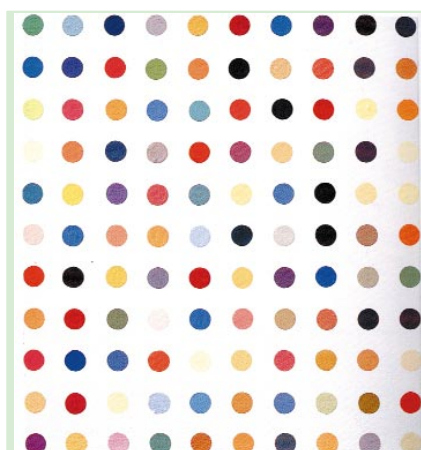
Some 45 years after Hopkins' address, Joseph Fruton wrote *Molecules and Life* (Wiley, 1972), which documented some of the history of the new frontier between organic chemistry and biology. That book (now out of print) forms the basis for *Proteins, Enzymes, Genes*.

The broad scope of Fruton's book makes it an ambitious undertaking, surely requiring a lifetime of devoted reading and research. And this is what Fruton has obviously given to the task, as evidenced by 248 pages of notes, references and bibliography (in small print), following 494 pages of text. Nothing like this can be found in the 1972 book. It is an invaluable scholarly resource and may be the most important feature of the new edition, the best justification for buying the new book even if one already has the old one. There is also a subject index in the new edition, frustratingly absent in the old.

Many sections of the older book have been transferred verbatim, but there is also considerable rewriting and new material that attempts to bring the subject into the present decade. The author has included two chapters that might be termed "historiography and philosophy of science": practitioners in these disciplines may not be altogether pleased with his robust views and undisguised dislike of some approaches to the history of science.

Turning to the text itself, it must be said that Fruton is uneven in his treatment of the broad range of subject matter that he has chosen. The best part is undoubtedly in the area of organic chemistry and its influence on the solution of biological problems — metabolism, energy transduction and modern molecular genetics. The interplay between physical chemistry and biology is less well covered, with the exception of the structural information obtained from X-ray crystallography. The vast amount of information about the size, shape and electrochemistry of proteins obtained using the techniques of solution physical chemistry is virtually ignored. Only six lines are given to one of the great landmarks in protein chemistry: the development of moving-boundary electrophoresis by Arne Tiselius and its exploitation for the separation of multiple discrete components from blood plasma. But a page or two later, two full pages are devoted to Dorothy Wrinch's unacceptable cyclol hypothesis, a brief episode in history that left no trace on subsequent thinking.

In similar vein, Fruton sometimes seems



Alternative structure catalyses art market

Artist Damien Hirst has found a more lucrative way of representing molecules than have most structural biologists. Carbon monoxide (above) is featured in *This is Modern Art* by artist and commentator Matthew Collings (Weidenfeld & Nicolson, £20), an eye-opening tour around contemporary art.

to go out of his way to defend the indefensible. Examples are the notorious Emil Abderhalden and his diketopiperazine hypothesis, Max Bergmann and Carl Niemann's numerology with respect to amino-acid composition, and Fruton's own initial scepticism (surely by now forgettable) of the Watson-Crick hypothesis.

Despite these criticisms, there are many valuable contributions in Fruton's book. The description of the growth of various scientific institutions throughout Europe and the United States is particularly useful — a source of information not readily available elsewhere. Similarly, the account of the discoveries of individual steps in metabolic pathways, creating order out of the confusion of the early twentieth century, gives a clear and detailed picture of the tremendous advances in organic chemistry that led to our current state of biochemical knowledge. This particular section would be useful reading for biochemistry students who are often overwhelmed by their first exposure to the myriad reactions going on inside the cell and who might profit from a step-by-step account of just how they were elucidated — including more than a few false steps along the way.

In summary, if you want a rich source of references, this may well be your book. But it fails to convey the sense of excitement that the discoveries of previous generations evoked. It is not meant to be a book that entertains as it teaches.

Charles Tanford and Jacqueline Reynolds are at Tarlwood, Back Lane, Easingwold, North Yorkshire YO6 3BG, UK.

Biology's structurally sound foundations

Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding

by Alan Fersht

W. H. Freeman: 1999. 614 pp. \$47, £27.95

Gregory A. Petsko

Genome sequences now accumulate so quickly that, in less than a week, a single laboratory can produce more bits of data than Shakespeare managed in a lifetime, although the latter make better reading. With this flood of data comes a demand for an understanding of the three-dimensional structure and function of the proteins encoded by these genes. But while sequencing genes is easy, it is not so easy to determine a gene product's function or the mechanism by which it acts.

Two seemingly unrelated subjects — protein folding and enzymology — are among the fields being enlisted to aid in this enormous task: the former in the hope that we will eventually be able to deduce the structures of proteins from their sequences alone; the latter because it attempts to explain how enzymes work by combining organic and inorganic chemistry with protein structure. In this book, one of the leading practitioners of both fields sets forth the physical-chemical principles that govern protein folding and enzyme action in a summary of more than 20 years of intense investigation.

A treatment of both enzyme catalysis and protein folding would usually be a multi-author text, edited by at least one expert from each field. But Alan Fersht has been a pioneer in both areas, giving this book a unique and coherent perspective. Although it has a new title and much new material, this book is really an updating and expansion of the author's classic text, *Enzyme Structure and Mechanism* (W. H. Freeman, 1985). This is a plus, because that book was a model of clear and concise exposition, and much of the original material has been retained, particularly in the chapters on chemical catalysis and enzyme reaction rates. Yet in the 14 years since the last edition of that book was published there has been an explosion of information about protein structure and its relation to function, driven largely by X-ray crystallography, nuclear magnetic resonance and protein engineering.

Quite properly, Fersht starts with protein structure. He reviews what is known about protein domains and higher levels of organization, not encyclopedically, but with selected examples. Although I would have chosen different examples in some cases, these mostly serve their purpose well. The chapter

on active-site-directed inhibitors is excellent from the chemical point of view, but could have benefited from more examples of structure. For instance, in the discussion of inhibitors of pyridoxal phosphate-linked enzymes, the illustrations are of vinyl glycine and β -chloroalanine, the mechanisms for which are still not definitively established, instead of cycloserine, for which they are.

Although the important topics of allosteric enzymes, molecular switches and motor proteins cannot be covered in great depth in a book of this sweeping scope, the general principles are clearly expounded and the examples used are excellent. Similarly, in the discussion on the use of binding energy in catalysis — often a confusing topic — Fersht achieves admirable clarity. In a new section, he then presents a number of case studies in protein engineering and enzyme structure and mechanism.

The book ends with three new chapters describing the results of protein-engineering studies on the energetics of protein structure, and the pathways by which some selected proteins fold. This is the best treatment I have read of this important topic. Unfortunately, the evidence suggests that there is no universal mechanism for protein folding, which tends to make every detailed study of the folding of a given protein *sui generis*. Fersht does not skirt this issue, but it seems that studies of the folding pathways of individual proteins are unlikely to help with the problem of going from a protein's sequence to its structure. Purely computational methods and comparison with other sequences and structures ('inverse folding') remain the best hopes for a practical solution.

A large part of the book, especially in the last few chapters, is a discussion of the author's own work. But, given his achievements, no book on mechanistic enzymology or protein folding could claim to be comprehensive without such discussion. Anyone attempting to do functional genomics will find this book invaluable, not only as a source of examples of how to do functional studies carefully, but also as a guide to the basic principles of how proteins work.

One complaint: my copy contained a number of references that did not match their citation in the text. And in the next edition, it would be useful to have a section on Web-based resources for protein structure, molecular biology and biochemistry. A modern text needs them.

Gregory A. Petsko is at the Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02454-9110, USA.

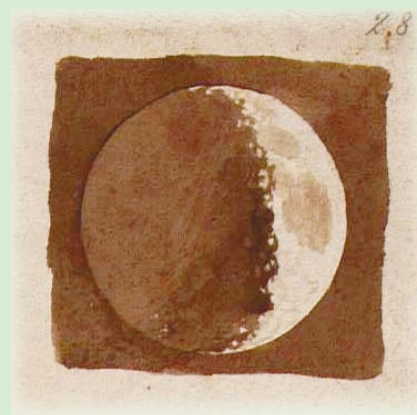
More on proteins

Introduction to Protein Structure, 2nd edn

by Carl Branden & John Tooze

Garland, £55, \$72.95 (hbk); £29.95, \$47.95 (pbk)

Science in culture



Maculate moons

Galileo and the lunar mountains

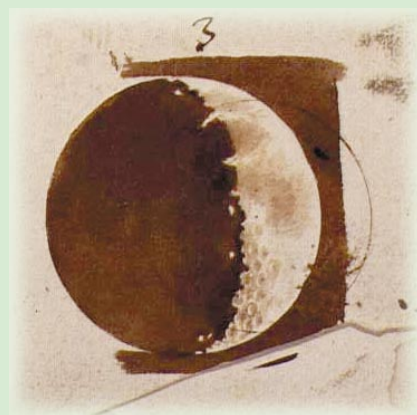
Martin Kemp

We all have a powerful tendency to see what we want to see. And shifting our perceptions often takes a huge effort. When Galileo trained his improved 'spyglass' on the Moon in the winter of 1509, he became convinced that "sane reasoning" could reach no other conclusion than that the Moon "is full of prominences and cavities similar to, but much larger than, the mountains and valleys spread over the Earth's surface". But he had reckoned without the powerful grip of those philosopher-theologians who wished to sustain their perception of the heavens as 'immaculate'.

For Galileo the argument was as plain as the nose on one's face — or as the Apennines that provide the spine of central Italy. Looking at the changing borderline between the lit and shaded hemispheres, he observed features that behaved like the shining and shaded ridges of mountains flanking valleys on Earth. Bright points sprouted and grew within the shadowy border, like earthly mountains receiving the rays of the rising Sun. Working out the optical geometry of the most prominent spots in terms of the passage of light across a rough sphere, Galileo calculated that the most prominent mountains were no less than four miles high.

But not everyone saw the same thing. Thomas Harriott in England had already viewed and drawn only a "strange spottednesse" with his low-grade instrument, while Christopher Clavius and his fellow 'mathematicians' in Galileo's Rome reported that it was "more probable that the surface is not uneven, but rather that the lunar body is not of uniform density and has its dense and rarer parts". Roman Jesuits were committed to the translucent immaculacy of the Moon, in accordance with the Song of Songs in the Bible, where the Virgin's pure beauty is identified with the Moon — "*pulchra ut luna*". Accordingly, they condemned the "filth of opacity" implied by Galileo.

Why did Galileo see and represent so clearly? The quality of his telescopes was important, as



was his conceptual freedom from dogmatic theology. But an essential ingredient was his ability to articulate his scrutiny and graphic record through a sophisticated understanding of the motion of light on bodies with uneven surfaces. As an accomplished draftsman and an aficionado of perspective, he was fully acquainted with the science of cast shadows outlined in textbooks such as Albrecht Dürer's *Instruction in Measurement* (1528) and Daniele Barbaro's *The Practice of Perspective* (1569).

As a member of the artists' Accademia del Disegno in Florence and a friend of the leading artist Ludovico Cigoli, Galileo was also aware of the tradition, inaugurated by Leonardo da Vinci, of minutely systematic observation and the depiction of light in a landscape according to the angles and direction of the Sun relative to the observer. Cigoli's own treatise on perspective quoted from Leonardo's unpublished writings. When Cigoli came, in 1610–12, to portray the "Virgin of the Immaculate Conception" in the dome of the Capella Paolina in Santa Maria Maggiore in Rome, he portrayed Mary standing on a pitted and maculate Moon rather than the smooth crescent of convention. In his defence, he could have pointed to an earlier tradition which justified the placement of the Moon below Mary's feet on the grounds that its patchy and inconstant appearance testified to its lowly status in the heavens.

Yet, for all his hard looking and knowing draftsmanship, the details of Galileo's cratered Moons are difficult to align precisely with actual features. With a field of view of less than half the Moon's diameter, and the inevitable difficulties of charting details without an existing framework, Galileo has, in effect, shown what happens as sunlight skates over the lunar landscapes rather than made a precise map. His purpose was to show the Moon as Earth-like. He sees what he wants to see and persuades us to do the same.

Martin Kemp is in the Department of the History of Art, University of Oxford, 35 Beaumont Street, Oxford OX1 2PG, UK.

Moral calculus and the bomb

Targeting civilians, not using atomic weapons, was the moral watershed.

Kurt Gottfried

The moral dilemmas thrown up by science are starkly illustrated by the discovery of nuclear fission. With the impact of science on society certain to increase in the next millennium, what can we learn from the story of nuclear weapons?

Since 1945 there has been constant debate as to whether the killing of nearly 200,000 Japanese civilians with nuclear weapons was morally justified. Given that by that time the creation of nuclear weapons was inevitable, perhaps the greater tragedy is that their birth could have come sooner, and saved far more lives than were extinguished in Hiroshima and Nagasaki.

It was not a foregone conclusion that the atomic bomb would only become available as late as August 1945. My colleagues Hans Bethe and Robert Wilson, the wartime heads of the theoretical and experimental nuclear physics divisions at Los Alamos, believe that a year was lost because the United States failed to build upon ground-breaking work carried out in England.

Early in 1940, Otto Frisch and Rudolf Peierls in Manchester wrote the memo that showed, for the first time, that the critical mass for ^{235}U is astonishingly small, and that large-scale separation of this isotope might be feasible. Leading scientists, who reported to Churchill's War Cabinet, refined these ideas and by the year's end were convinced that a bomb could be built within several years. By contrast, fission research in the United States received paltry government support. Only after repeated entreaties from British physicists did Roosevelt, in October 1941, decide to commit a major effort to the building of the bomb.

So, a good portion of a year — if not actually a whole year — could have been saved. Setting detailed speculations aside, consider the consequences had the bomb been available at various earlier times.

In the Pacific theatre, six months would have made an enormous difference. The systematic fire bombing of the great Japanese cities only began in March 1945, and killed more people in Tokyo alone than were to die in Hiroshima. Okinawa was captured in the spring of 1945 with 20,000 US and 110,000 Japanese military fatalities.

Many believe that the Western Allies would not have used the bomb against Europeans. There is no basis for this speculation. We had no qualms about burning alive some 135,000 civilians in Dresden, even though this beautiful city had no military value and



Atomic desolation? No, this was Tokyo in 1945, after saturation bombing with non-nuclear weapons.

was about to fall to the Red Army. Surely the bomb would have been used against the Nazi state, and, if feasible, against Hitler himself. After Stalingrad and the Normandy landings, there were many in the German High Command who knew the war was lost; Hitler could not have survived politically in the face of atomic attack. And repeated attacks would have been possible, because by the end of the war the Manhattan Project could produce one Nagasaki-type plutonium bomb roughly every two weeks.

Had the bomb ended the European war in December 1944 — eight months before it fell on Hiroshima and two months before the Yalta conference that divided up Europe — the Soviet army would not yet have held Warsaw or Budapest, and would have still been far from Prague. Tens of millions would not have had to endure 44 years of Soviet

occupation. The lives of an enormous number of soldiers on the Eastern front, and a smaller number on the Western front, would have been saved. A significant portion of the millions who died in Nazi death camps would have survived. Even if the war in Europe had ended in April 1945, instead of in May, the benefits would still have been substantial.

The profound moral divide that was crossed by the great democracies was their considered decision to systematically kill civilians by strategic bombing. Once that became routine, the use of the atomic bomb for the same purpose followed inexorably. The subsequent deployment of grotesque thermonuclear arsenals, that put whole societies at risk, and the adoption of military postures that reduced decision times to less than 30 minutes posed a far graver affront to morality and sanity, and proved to be virtually unavoidable.

The use of the bomb in the Second World War illustrates the obvious in the starkest terms: moral calculus does not lead to unambiguous answers. And the whole history of the nuclear age shows that the combination of new science with the abandonment of a profound moral principle — in this case that civilians should not be military targets — can lead to awesome dangers that could not have been imagined at the outset. □

Kurt Gottfried is at the Laboratory of Nuclear Studies, Cornell University, Ithaca, New York 14853-5001, USA

The combination of new science with the abandonment of a profound moral principle can lead to dangers that could not have been imagined

Birds of a feather lek together

Paul W. Sherman

A lek looks like a winner-takes-all competition between males to attract females. But appearances could be deceptive, and the males might be a family group cooperating to their mutual evolutionary advantage.

The lek is nature's version of a singles bar. A group of males aggregates at a traditional site, where they perform intricate vocal, visual or chemical displays to attract receptive females. Lekking arenas contain no resources valuable to females and males give no parental care, so females choose mates by comparing males' physiques and displays, or copying choosy females. On most leks only a few, extremely attractive males do nearly all of the mating — so why do subordinates bother joining them? Two studies, one of black grouse in Finland, published in *Proceedings of the Royal Society*¹, and the other of free-ranging peacocks at Whipsnade Park in the United Kingdom, on page 155 of this issue², reveal that males lek with their kin. Males that are apparently unsuccessful may gain an evolutionary advantage by boosting the mating success of family members. Petrie *et al.* also show that peacocks recognize brothers by 'phenotype matching' — comparing other males with themselves.

Lekking is rare but taxonomically widespread, occurring mainly in insects and birds (about 200 species), but also in some mammals, amphibians and fishes³. Three hypotheses have been proposed to explain why males aggregate: the hotspot model (clusters form near places females frequently visit), the hotspot model (individuals cluster around attractive males to increase their chances of being noticed), and the female-preference model (males cluster because females like to visit groups, where they can choose a mate quickly and safely). Tests of each hypothesis have found support in some species, but not others³.

All three hypotheses predict that males should join leks to increase their mating opportunities. Indeed, in some species larger leks do attract proportionally more females. However, in many species mating success per male declines as lek size increases^{4,5}, raising the question of why males keep joining up. Either they have no better options, or they are gaining reproductive benefits in some other way.

For example, if larger, cooperatively displaying groups make leks more attractive, subordinate males might increase their inclusive fitness — the number of additional genes their behaviour enables kin to pass to the next generation — by joining leks dominated by their relatives^{6,7}. For example, when subordinates are brothers or sons of the



Figure 1 Display of unity? Petrie *et al.*² found that male peacocks choose related birds as lek mates. What's more, it seems that males don't need to learn who their relatives are to be able to recognize them.

alpha male, his matings will conceive their nieces and nephews or full- and half-siblings, respectively. The benefits of helping closely related dominants to attract more females may outweigh the subordinate males' own meagre mating opportunities.

A critical prediction of this kin-selection hypothesis is that males will be more related within than among leks. The first species tested was the long-tailed manakin, a bird that lives in Central American rainforests⁸. Males display cooperatively in pairs, but the dominant of the two wins 99% of the copulations. However, analyses of DNA microsatellites revealed that displaying pairs were no more likely to be related than any two randomly chosen birds. (Molecular techniques, such as DNA fingerprinting and microsatellite analysis, have been a godsend for behavioural ecologists, as they allow levels of relatedness to be measured directly — information that field observations alone cannot reveal.)

But the new studies^{1,2} have revived the kin-selection hypothesis. In the grouse, Höglund *et al.*¹ used DNA microsatellites to infer the existence of significant genetic differentiation among five winter flocks of males (but not females), and among 15 leks composed of males from the same and different winter flocks — in other words, males associate with family members. In the peacocks, Petrie *et al.*² used DNA fingerprinting to infer that relatedness was significantly higher within each of four leks than between them; average relatednesses for lek-mates approximated those of half-brothers. In both

black grouse and peacocks, larger leks — consisting of up to 20 males — attract more females and male mating success is skewed.

So, how did related males end up on the same arena? For the grouse, genetic structuring of winter flocks and leks was attributed to natal philopatry — the tendency for individuals to remain in the area where they grew up — although differentiation among leks from the same winter flock "suggests that some active kin-recognition mechanism may be at work"¹.

For the peacocks, the serendipitous extension of a sexual-selection experiment⁹ revealed that natal philopatry cannot be the explanation. In 1991, Petrie and her colleagues removed eight breeding peacocks that differed in their attractiveness to females from Whipsnade Park and moved them to Norfolk, over 100 km away. Each was penned with four randomly chosen peahens. Their eggs were collected daily, and the chicks hatched in isolation in an incubator. Chicks were ringed and reared in groups from the same and different pens. Presumably, therefore, they could not learn from their social environment who their relatives were. Early in 1992, 96 yearlings (three from each female) were released in Whipsnade Park.

In 1995, when the 19 surviving four-year-old males established permanent display sites, their territories were mapped by observers who were unaware of the birds' relatednesses. Surprisingly, there were highly significant tendencies for (full- or half-) brothers to display closer to each other than to non-relatives. Brothers were also nearest display-neighbours far more often than would be expected from chance.

There are three ways that the peacocks could have recognized their siblings¹⁰. First, related birds might prefer similar micro-environments, even in the homogeneous, non-native parkland. But the released males did not display near their fathers' ex-display sites, as would be expected if such a preference were heritable. Second, birds might learn to recognize the young they grew up with (normally their siblings), and later use this mental image to match the birds they encountered. But males that were reared together did not lek together. Third, males might learn their own physical features and later associate with phenotypically similar birds. Such self-referent phenotype matching¹¹, which Dawkins¹² dubbed the 'armpit

MARION PETRIE



100 YEARS AGO

Many observers in different countries, noticing the fact that malaria is most prevalent at the most active period of mosquito life, have attributed malaria to the agency of this insect. Dr. Patrick Manson, in 1894, first brought the subject forward in England ... Bignami and Bastianelli, who had been trying unsuccessfully to infect a man by allowing mosquitoes to bite him, attributing their want of success to the use of the wrong kind of mosquito, and, acting on the observations of Grassi, tried again with some mosquitoes imported from the malarious district. This time they succeeded in infecting the man with malaria of the same type that prevailed in the district from which the mosquitoes came. ... Whether the *Anopheles* can be extirpated from a locality, and by what means, will be the problem for scientific workers resident abroad to settle; fortunately they seem to be confined to small areas, so the suggestion of Ross to draw off the water from stagnant pools may not be so hopeless a task as it would at first appear.

From *Nature* 7 September 1899.

50 YEARS AGO

For the woman, Dr. Turquet saw two possibilities fraught with anxiety and frustration and therefore likely to add to the sum of aggression in society. One is her entry into an occupational life in which her opportunities compare unfavourably with a man's. If, on the other hand, she prefers the older domestic role with its exemption from the need to compete on the male pattern, she then faces the anxiety of choosing and securing a husband, on whose adequacy her own social status will largely depend, and to whom she will lose some of her individual identity. In either case the woman meets an atmosphere of masculine superiority which contrasts sharply with her childhood experience of the mother's seeming superiority in the home. Aggression results from the inferiority which she feels in such an atmosphere. "Hence," said Dr. Turquet, "ambivalent feelings towards motherhood, the declining birthrate, and an unconscious desire to reject the more fundamental aspects of the feminine role".

From *Nature* 10 September 1949.

Many more extracts like these can be found in *A Bedside Nature: Genius and Eccentricity in Science, 1869–1953*, a 266-page book edited by Walter Gratzer. Contact Lisa O'Rourke. e-mail: lorourke@nature.com

effect, could allow males with the same father to recognize one another, whether they were reared together or apart, as often occurs in species where males mate with many females.

I believe that this is the most likely explanation. However, confirmation requires identifying what heritable cues males use (for example, calls, plumage or odour), altering them experimentally, and observing whether manipulated males indeed prefer similar but unrelated lek-partners.

Petrie and colleagues' results² are important for several reasons. First, they (and Höglund *et al.*¹) suggest that lek-joining in some species is best analysed by considering inclusive fitness, not just male–male competition. Second, although phenotype matching has been observed in many invertebrates and vertebrates¹⁰ (most recently in chimpanzees¹³), this is the first report for a lekking species. Third, the likelihood of self-referencing in peacocks should galvanize studies of this intriguing, yet controversial^{10,11,14},

kin-recognition mechanism, particularly among species in which social learning is an inadequate or misleading guide to relatedness.

Paul W. Sherman is in the Department of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853, USA.

e-mail: pws6@cornell.edu

- Höglund, J., Alatalo, R. V., Lundberg, A., Rintamäki, P. T. & Lindell, J. *Proc. R. Soc. Lond. B* **266**, 813–816 (1999).
- Petrie, M., Krupa, A. & Burke, T. *Nature* **401**, 155–157 (1999).
- Höglund, J. & Alatalo, R. V. *Leks* (Princeton Univ. Press, 1995).
- Deutsch, J. C. *Behav. Ecol. Sociobiol.* **34**, 451–459 (1994).
- Widemo, F. & Owens, I. P. F. *Nature* **373**, 148–151 (1995).
- Watts, C. R. & Stokes, A. W. *Sci. Am.* **224**, 112–118 (1971).
- Kokko, H. & Lindström, J. *Proc. R. Soc. Lond. B* **263**, 919–923 (1996).
- McDonald, D. B. & Potts, W. K. *Science* **266**, 1030–1032 (1994).
- Petrie, M. *Nature* **371**, 598–599 (1994).
- Sherman, P. W., Reeve, H. K. & Pfennig, D. W. in *Behavioural Ecology*, 4th edn (eds Krebs, J. R. & Davies, N. B.) 69–96 (Blackwell, Oxford, 1997).
- Sherman, P. W. *Ethol. Sociobiol.* **12**, 377–386 (1991).
- Dawkins, R. *The Extended Phenotype* (Freeman, San Francisco, 1982).
- Parr, L. A. & deWaal, F. B. M. *Nature* **399**, 647–648 (1999).
- Alexander, R. D. *Ethol. Sociobiol.* **11**, 241–303 (1990).

Nanotechnology

Synthetic molecular motors

Anthony P. Davis

The construction of miniature, 'nanoscale' machines is a goal of modern science and technology, inspired by Richard Feynman's remark that "There's plenty of room at the bottom"¹. Chemists, by the nature of their discipline, are already at the bottom, manipulating the smallest entities that have complex shapes (molecules), and which can therefore be used as engineering components. While engineers and physicists explore the top-down approach to nanoscale engineering through lithography and scanning probe microscopy, chemists are well placed to pursue the bottom-up strategy, whereby molecular-scale components are created using chemical synthesis and then self-assembled into devices by pre-programmed intermolecular forces².

Among the more interesting challenges in this area is the design and synthesis of 'molecular actuators', molecules that can undergo changes in shape in response to external stimuli and thereby, in principle, perform mechanical work. To date, most research has concentrated on two-state systems, ranging from classical *cis–trans* isomerism to more elaborate 'rotaxanes' and 'catenanes' (Fig. 1)^{3,4}, and biomolecular constructs, such as a device based on the transition of right-handed to left-handed DNA⁵. These systems, in which movement is driven by chemical, electrochemical or photochemical forces, are best described as molecular

switches or shuttles, and they have great potential in, for example, molecular-scale information processing. However they are not capable of the continuous, unidirectional

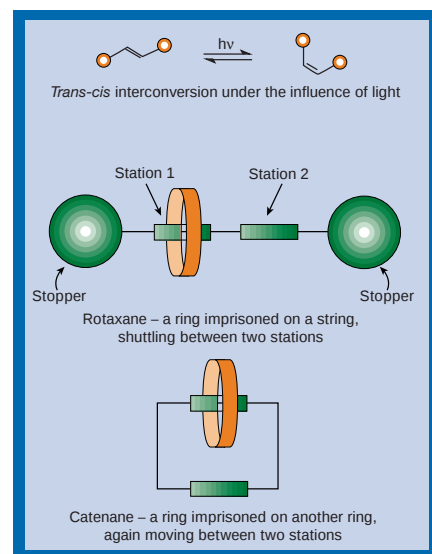


Figure 1 Two-state molecular systems.

Established molecular actuators include systems capable of *cis–trans* isomerism, where groups lie on the same (*cis*) or opposite (*trans*) sides of a double bond, and more complex structures such as rotaxanes and catenanes. The rings in the rotaxanes and catenanes may be driven between stations by chemical, electrochemical or photochemical input.

al motion we would expect of a true molecular motor. Now, on pages 150 and 152 of this issue, Kelly *et al.*⁶ and Koumura *et al.*⁷ report two different routes towards this seductive goal.

The design of Kelly *et al.*⁶ is based on a previous attempt to construct a 'molecular ratchet'. In this earlier work, the rotation of a three-pronged 'wheel' was hindered by a curved 'brake' (Fig. 2a), whose asymmetrical shape was intended to favour rotation in one particular direction⁸. Such unidirectional motion, driven only by ambient thermal energy, would have violated the second law of thermodynamics⁹, and unsurprisingly failed to occur. Careful NMR measurements showed that the ratchet rotated in either direction with equal probability.

Kelly *et al.* have now modified their system, using the asymmetry of their brake to produce chemically driven (and therefore thermodynamically allowed) motion in one direction. Functional groups are attached to the brake and one spoke of the wheel, allowing the two to be linked covalently. The linkage pulls the molecule into a strained, high-energy conformation, so that it becomes energetically favourable for the spoke to slip under the brake. Cleavage of the linkage returns the system to its original chemical state, but with the wheel having undergone one-third of a rotation. The system is not a true molecular motor, as no provision is made for further movement, but it does demonstrate a method by which chemical energy may be transduced, on a molecular scale, into unidirectional rotary motion. Understanding this and similar processes may be relevant to natural molecular motors, such as ATP synthase^{10,11}, which are also powered by chemical reactions.

A critical aspect of the above system is its chirality (handedness), without which a direction of rotation could not be defined, let alone pursued. The design of Koumura *et al.*⁷ also obeys this principle. Their system is an alkene molecule (that is, it contains a carbon-carbon double bond), and uses the photochemical *cis-trans* interconversion illustrated in Fig. 1. But whereas simple allenes are planar and achiral (that is, superimposable on their mirror images), the system of Koumura *et al.* has a complex structure which prevents planarity, introduces chirality and results in four distinguishable states, two *trans* and two *cis* (Fig. 2b). As expected, both *trans-cis* interconversions can be achieved by illumination with ultraviolet light of an appropriate wavelength.

The singular feature of this system is that, for subtle structural reasons, one of each pair of *trans*- and *cis*-isomers is more stable than the other. Provided the temperature is high enough to overcome the activation barrier between the two *trans* or *cis* isomers, the more stable of the pair is formed irreversibly from its partner. So, light-driven creation of

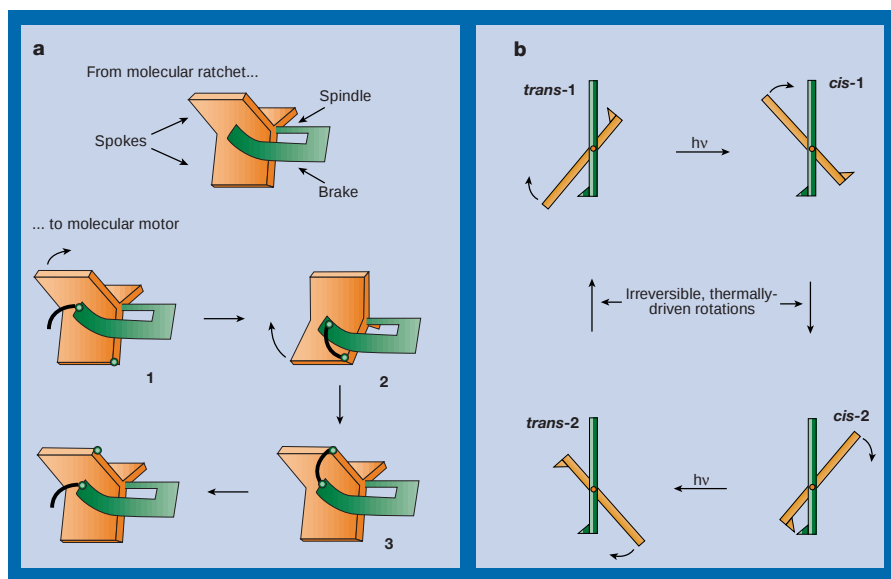


Figure 2 Unidirectional motion produced by nanoscale rotors. **a**, In the chemically driven system created by Kelly *et al.*⁶, the ratchet design (top) is modified so that a link can be made between the brake and one of the spokes. Making the link (1 $\rightarrow$ 2) pulls the rotor into a strained, high-energy position. Thermal energy is now sufficient to carry the spoke past the brake, allowing the rotor to regain its favoured situation as in 3. Cleavage of the link restores the original position, except that the rotor has moved through 120°. **b**, The light-powered system created by Koumura *et al.*⁷, viewed down the axis of a central carbon-carbon double bond that links two identical halves of an alkene molecule. The double bond is trying to establish a planar structure — that is, to make the two halves line up. But they are prevented from doing so by bulky substituent groups, resulting in a twisted geometry with four distinct states. The barrier between *cis* and *trans* forms is high, but may be overcome by ultraviolet irradiation. Asymmetric substituents (triangles) cause energy differences between the two *cis* and two *trans* isomers, such that *cis*-2 is more stable than *cis*-1, whereas *trans*-1 is more stable than *trans*-2. At appropriate temperatures, the thermally driven *cis*-1 $\rightarrow$ *cis*-2 and *trans*-2 $\rightarrow$ *trans*-1 conversions take place irreversibly. The net result is continuous rotation in the presence of ultraviolet light.

the less stable isomer of each pair is followed by thermal decay into the more stable isomer, resulting in overall rotation (Fig. 2b). The authors have followed this cycle stepwise by performing the photochemical conversions at low temperatures, which slows the *trans-trans* and *cis-cis* interconversions so that each of the four species can be observed in sequence. However, at higher temperatures there seems no reason why the system should not rotate continuously under ultraviolet irradiation, and so be seen as a genuine, light-powered molecular motor.

Many problems remain before the principles embodied in these molecular motors can be incorporated in real, useful machines. The rotors must be coupled to their surroundings, and the self-assembly processes needed for nanoscale construction still need to be worked out. Nonetheless, the results of these two groups provide further confirmation that molecular-scale engineering is slowly edging towards reality. The difficulties should not be underestimated; molecular design is still an uncertain business, and chemical synthesis can be lengthy and painstaking. Indeed, an engineer may be amazed that the 'partial motor' of Kelly *et al.*, containing only 78 atoms,

required over four years to construct. It should not be forgotten, though, that synthesis is a massively parallel manufacturing process; each mole of product corresponds to $\sim 6 \times 10^{23}$ discrete 'nanodevices'. Both groups have probably made at least 10^{19} units of their respective systems, and could scale up by several orders of magnitude if required. The bottom-up approach is certainly challenging, but may ultimately have economics on its side.

Anthony P. Davis is in the Department of Chemistry, Trinity College, Dublin 2, Ireland.
e-mail: adavis@tcd.ie

1. Feynman, R. *Eng. Sci.* **23**, 22 (1960).
2. Philp, D. & Stoddart, J. F. *Angew. Chem. Int. Edn Engl.* **35**, 1155–1196 (1996).
3. Balzani, V., Gomez-Lopez, M. & Stoddart, J. F. *Acc. Chem. Res.* **31**, 405–414 (1998).
4. Sauvage, J. P. *Acc. Chem. Res.* **31**, 611–619 (1998).
5. Mao, C. D., Sun, W. Q., Shen, Z. Y. & Seeman, N. C. *Nature* **397**, 144–146 (1999).
6. Kelly, T. R., De Silva, H. & Silva, R. A. *Nature* **401**, 150–152 (1999).
7. Koumura, N., Zijlstra, R. W., van Delden, R. A., Harada, N. & Feringa, B. L. *Nature* **401**, 152–155 (1999).
8. Kelly, T. R., Tellitu, I. & Sestelo, J. P. *Angew. Chem. Int. Edn Engl.* **36**, 1866–1868 (1997).
9. Davis, A. P. *Angew. Chem. Int. Edn Engl.* **37**, 909–910 (1998).
10. Noji, H., Yasuda, R., Yoshida, M. & Kinosita, K. *Nature* **386**, 299–302 (1997).
11. Walker, J. E. *Angew. Chem. Int. Edn Engl.* **37**, 2309–2319 (1998).

Cell biology

Lipid membranes shape up

Suzie J. Scales and Richard H. Scheller

Nervous transmission depends on the rapid fusion of neurotransmitter-laden vesicles with the presynaptic plasma membrane. The released neurotransmitter diffuses across the gap between two nerve cells (the synapse), while the non-cargo components of the fused vesicle are retrieved from the presynaptic membrane in a process called synaptic-vesicle recycling. The whole fusion and recycling process takes only about 40 seconds to complete¹.

On page 133 of this issue, Schmidt *et al.*² offer an exciting insight into the main pathway by which synaptic vesicles are recycled — a process called clathrin-mediated endocytosis (Fig. 1). Clathrin molecules assemble at the plasma membrane to form a shallow pit that invaginates and finally pinches off into a free vesicle, in a step known as fission.

A GTP-hydrolysing protein (GTPase) called dynamin is known to be involved in the fission step.

For a long time dynamin was thought of as a 'pinchase', akin to a boa constrictor³, forming rings around the neck of the budding vesicle and mediating its final constriction. But, earlier this year⁴, dynamin's GTPase activity was implicated in regulating downstream 'effector' molecules that cause vesicle fission. Schmidt *et al.*² now show that one such potential dynamin effector may be a molecule called endophilin I (previously known as SH3p4). The authors show that endophilin I is a lysophosphatidic acid acyl transferase (LPAAT), and suggest that it changes the shape of the membrane lipids so as to facilitate invagination.

Schmidt and Huttner⁵ have previously

shown that clathrin, dynamin and endophilin I are among the essential components for the formation of synaptic-like microvesicles (SLMVs) from invaginations of the plasma membrane. They did this by reconstituting the process in permeabilized PC12 cells using cytosol and ATP⁵, a similar assay to that developed by Kelly and colleagues⁶ for the formation of endosomal SLMVs. Endophilin I, which is a brain-specific protein, contains a region known as a src-homology 3 (SH3) domain. This domain interacts with the proline-rich region of dynamin, as well as with that of two other synaptic proteins, synaptotagmin and amphiphysin⁷⁻⁹. Schmidt and colleagues now show that endophilin I also has LPAAT activity, catalysing the formation of phosphatidic acid from lysophosphatidic acid (LPA; Fig. 2). They go to great lengths to show that purified (or recombinant) endophilin I binds its two substrates (LPA and fatty acyl CoA), and that the formation of SLMVs depends on its LPAAT activity.

How could endophilin I's LPAAT activity affect fission? Membrane phospholipids come in different shapes, depending on the relative sizes of their polar head-groups and hydrophobic fatty-acid tails. Viewed from the head-group end, phospholipids with a larger head than tail — such as LPA, which has just one saturated fatty-acid tail — are said to be inverted-cone shaped (Fig. 2). On the other hand, phospholipids with a wider tail than head, particularly those with an unsaturated fatty acid (such as phosphatidic acid) are cone shaped. Inverted-cone-shaped lipids favour outward bending, or positive curvature, of the membrane, such as is found in the outer (cytoplasmic) leaflet of a plasma-membrane bud. Cone-shaped lipids, by contrast, favour negative curvature (inward bending), as in the inner (exoplasmic) leaflet of the bud¹⁰.

Considering the cytoplasmic leaflet only (because endophilin I is found only on this side of the membrane), Schmidt *et al.*² propose that endophilin I changes the inverted-cone-shaped LPA (in the positively curved upper half of the 'S'-shaped bud on the left half of Fig. 1c) into cone-shaped phosphatidic acid (in the negatively curved lower half of the 'S'). This change may help the membrane to invaginate at the neck of the bud, an effect that was previously attributed to dynamin. This appealing hypothesis is strengthened by the observation that inverted-cone-shaped lipids inhibit — and cone-shaped lipids promote — fusion of liposomes¹⁰, as well as the formation of SLMVs documented by Schmidt and colleagues. Furthermore, the authors found that a form of endophilin I that lacks its dynamin-binding SH3 domain, but retains LPAAT activity, is defective in the formation of SLMVs.

Schmidt and colleagues go on to suggest that dynamin may recruit endophilin I to

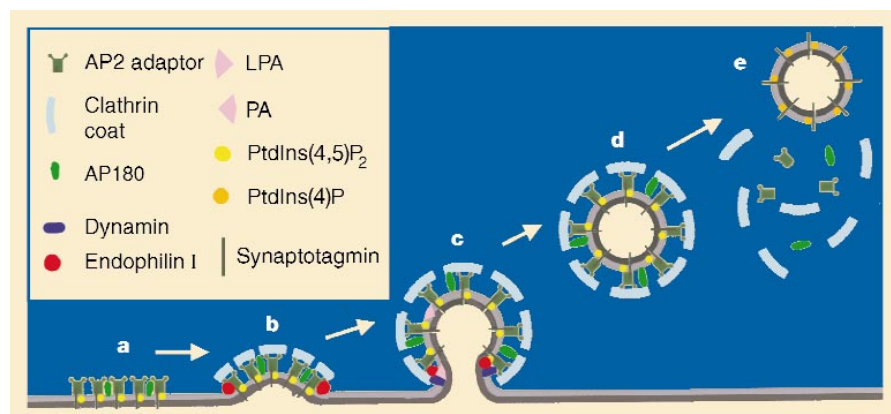


Figure 1 Clathrin-mediated endocytosis. a, Synaptotagmin and the AP2 adaptor complex are recruited to lipids such as phosphatidylinositol-4,5-bisphosphate PtdIns(4,5)P₂, and, along with AP180, recruit clathrin to form a shallow coated pit (b), which invaginates in a process facilitated by dynamin and, according to the results of Schmidt *et al.*², endophilin I (c). The coated vesicle pinches off (d) and dephosphorylation of PtdIns(4,5)P₂ may facilitate its uncoating (e).

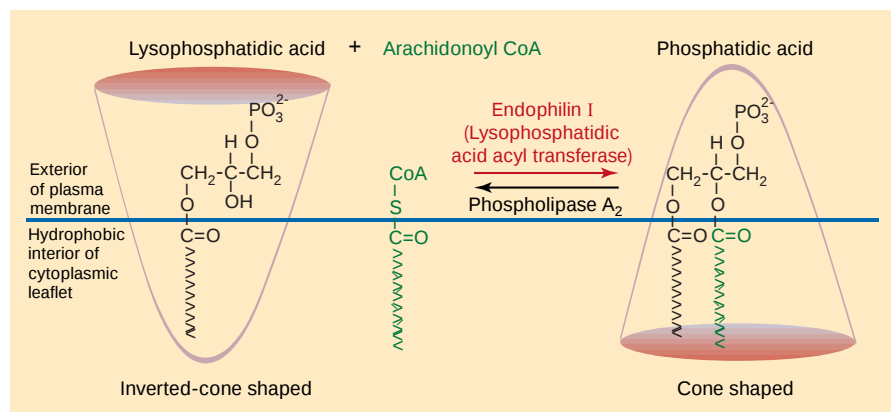


Figure 2 The lipid-transfer reaction catalysed by endophilin I. Endophilin I catalyses the addition of the unsaturated fatty acid arachidonate (from the acyl donor arachidonoyl CoA) to lysophosphatidic acid to form phosphatidic acid. This converts an inverted-cone-shaped lipid into a cone-shaped lipid, perhaps facilitating a change in membrane curvature.

the neck of the bud. However, endophilin I's SH3 domain interacts preferentially with synaptojanin over dynamin *in vitro*^{7,9}. This domain also binds transiently to amphiphysin⁸ and, perhaps, to other, as-yet-unidentified proteins containing proline-rich domains. So far, the authors have monitored only the end product of SLMV formation — the free vesicle. In other words, they don't yet have direct evidence that endophilin I affects membrane curvature. Indeed, Ringstad *et al.*¹¹ have performed electron microscopy on the presynaptic compartment of lamprey giant reticulospinal axons to show that anti-endophilin I antibodies inhibit the formation of clathrin-coated vesicles at the shallow-pit stage (Fig. 1b), rather than at the more deeply invaginated stage (Fig. 1c). Moreover, such pits lack dynamin collars¹¹. So endophilin I may, in fact, act upstream rather than downstream of dynamin. However, when Ringstad and colleagues studied coated pits that are more analogous to the SLMVs studied by Schmidt *et al.*, they found that these pits were more deeply invaginated (although they still lacked dynamin collars). This may mean that endophilin I acts at more than one step.

Although Schmidt and colleagues' results are consistent with the idea that endophilin I's LPAAT activity induces membrane curvature, there are alternative explanations. For example, perhaps an unknown pinching protein is recruited to either endophilin I itself or to the modified membrane lipids. Or, maybe endophilin I controls membrane tension via the interactions of synaptojanin or dynamin with the underlying actin cytoskeleton. Finally, it is conceivable that endophilin I could recruit synaptojanin downstream of dynamin to mediate the uncoating of clathrin-coated vesicles after they have been pinched off. These models can now be tested, because Schmidt *et al.* have changed the shape of future endocytosis research.

Suzie J. Scales and Richard H. Scheller are at the Howard Hughes Medical Institute, Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California 94305-5345, USA.

e-mail: sjscales@cmgm.stanford.edu

1. Klingauf, J., Kavalali, E. T. & Tsien, R. W. *Nature* **394**, 581–585 (1998).
2. Schmidt, A. *et al.* *Nature* **401**, 133–141 (1999).
3. Kirchhausen, T. *Nature* **398**, 470–471 (1999).
4. Sever, S., Muhlberg, A. B. & Schmid, S. L. *Nature* **398**, 481–486 (1999).
5. Schmidt, A. & Huttner, W. B. *Methods: A Companion to Methods Enzymol.* **16**, 160–169 (1998).
6. Faúndez, V., Horng, J. T. & Kelly, R. B. *Cell* **93**, 423–432 (1998).
7. Ringstad, N., Nemoto, Y. & de Camilli, P. *Proc. Natl Acad. Sci. USA* **94**, 8569–8574 (1997).
8. Micheva, K. D., Ramjaun, A. R., Kay, B. K. & McPherson, P. S. *FEBS Lett.* **414**, 308–312 (1997).
9. de Heuvel, E. *et al.* *J. Biol. Chem.* **272**, 8710–8716 (1997).
10. Chernomordik, L., Kozlov, M. M. & Zimmerberg, J. *J. Membr. Biol.* **146**, 1–14 (1995).
11. Ringstad, N. *et al.* *Neuron* (in the press).

Astronomy

Supernova birth for a black hole

John Cowan

Black holes have long been regarded as mysterious or exotic objects. Although there is growing observational evidence to support their existence, there are still questions about how these objects form. In one possible scheme a massive star (more than 25 times the mass of the Sun, $M_{\odot}$) runs out of nuclear fuel and collapses under its large gravitational mass to form a black hole. Another, more complicated model proposes that a massive star first explodes, leaving behind an extremely dense neutron star less than 20 km in diameter. Some of the matter thrown off by this supernova explosion falls back onto the newly born neutron star, which eventually collapses to form a black hole (Fig. 1). On page 142 of this issue Israelian *et al.*¹ report observations that probe the formation of these exotic objects, and suggest that the supernova route is possible, at least for one black hole.

The search for black holes has mainly depended on measuring the gravitational masses of the (unseen) companions of stars in binary systems. The strong gravitational attraction of a black hole, in systems where the stars are physically close, results in material being pulled off the companion star (Fig. 1). The matter accreted by the black hole forms a disk that swirls around the hole with some material falling into it, rather like water down a drain. The gas orbiting near the black hole is hot enough to occasionally emit X-rays. From observations of periodic shifts in the orbit of the companion star, the mass of the compact object can be inferred. Neutron stars have a maximum estimated mass of less than 3 $M_{\odot}$, so masses in excess of 3 $M_{\odot}$ are a clear indication of the presence of a black hole². The Sun and the vast majority of stars will end their lives as white dwarfs with masses less than 1.4 $M_{\odot}$.

The X-ray binary GRO J1655–40, also known as Nova Scorpii 1994, contains a black hole with an estimated mass of 5.5–7.9 $M_{\odot}$ and a sub-giant companion star with a mass of 1.7–3.3 $M_{\odot}$ (refs 3,4). Israelian *et al.*¹ observed Nova Scorpii 1994 during a quiet X-ray phase using a high-resolution spectrograph to analyse the visible and ultraviolet light emitted by the companion star. They were able to measure the abundances of several heavy elements in Nova Scorpii 1994. In particular they found evidence of the so-called α -elements — O, Mg, Si and S — with abundances six to ten times higher than in the Sun. This enhancement is significant because these α -elements cannot have been produced in such quantities in the interior of the star.

Heavy α -elements are created by adding

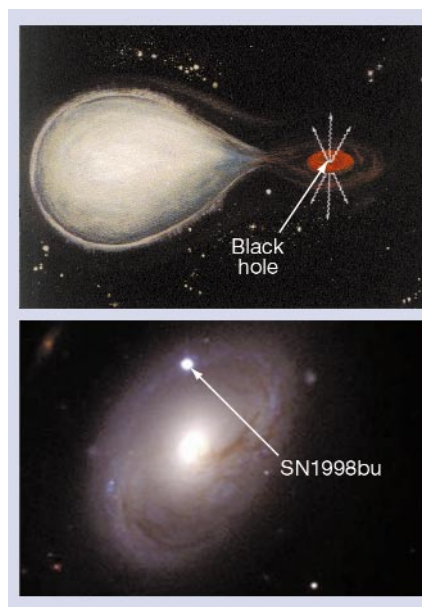


Figure 1 A black hole is born. Some stars explode so violently that for a few weeks the resulting supernova can outshine its parent galaxy (see bottom image). Above is an artist's impression of an X-ray binary system, in which matter is streaming from the companion star to the black hole. Hot gases in the accretion disk around the black hole occasionally emit X-rays. New observations of an X-ray binary by Israelian *et al.*¹ provide evidence that the black hole in that system was formed during a supernova explosion.

^4He nuclei, or α -particles, to the nucleus during the very last stages of the evolution of massive stars that will explode as supernovae. Elements produced by explosive oxygen burning, such as ^{16}O , ^{28}Si , ^{32}S , ^{36}Ar and ^{40}Ca , are created in the inner cores of these 25–40 $M_{\odot}$ massive stars at temperatures in excess of three billion Kelvin and in a matter of seconds⁵. Most normal, low-mass stars, such as the Sun, will never achieve such temperatures during their lifetimes and therefore are unable to synthesize α -elements.

Although there are other possibilities, the most logical explanation for the higher abundances of these α -elements in the companion star of Nova Scorpii 1994, and the one proposed by Israelian *et al.*, is that they are the result of the supernova explosion of the original massive star in this system. What is still not clear is why the supernova explosion did not disrupt or remove parts of the stellar atmosphere of the companion star. Nor is it clear exactly how the α -rich material was captured and retained by the companion.

Israelian and colleagues' prediction of the original mass of the supernova progeni-

NASA

NICHOLAS SUNTZE/CFR/CCTO

tor, based on the fact that the binary system was not totally disrupted during the explosion, is consistent with stellar evolution models^{5,6} of massive stars with 25–40 $M_{\odot}$. The expected yields of α -rich elements from explosive processing in these massive stars are also consistent with the observed abundances in Nova Scorpii 1994. The timing of the black hole formation can also be estimated. The detection of α -elements in the companion star suggests that only a small fraction of its mass could have been transferred to the black hole. Mass-loss rates in typical X-ray binaries suggest that the black hole in Nova Scorpii 1994 formed less than one million years ago, and has not increased greatly in mass since then.

There are, of course, still a number of unanswered questions. Is the formation history of the black hole in Nova Scorpii 1994 typical? We don't know what fraction of black holes goes through an intermediate supernova stage. Supernovae are relatively rare—a massive star is thought to explode in our Galaxy about every 100 years. So stars

more massive than 40 $M_{\odot}$ may collapse directly to form black holes or there may be cases where both a black hole and a supernova form simultaneously. Can we detect the presence of α -elements, the signature of such supernova explosions, in black hole companions in other X-ray binary systems (Cygnus X-1, for example)? If so, we could then use those observations to understand more about the details of element formation in these supernova explosions. Clearly many more observations are needed to answer these questions, but we now have the first convincing evidence that at least some black holes are created by supernovae. ■

John Cowan is in the Department of Physics and Astronomy, University of Oklahoma, 440 West Brooks Street, Norman, Oklahoma 73019, USA. e-mail: cowan@mail.nhn.ou.edu

1. Israeli, G., Rebolo, R., Basri, G., Casares, J. & Martin, E. L. *Nature* **401**, 142–144 (1999).
2. Kalogera, V. & Baym, G. *Astrophys. J.* **470**, L61–L64 (1996).
3. Orosz, J. & Bailyn, C. *Astrophys. J.* **477**, 876–896 (1997).
4. Shabaz, T. *et al. Mon. Not. R. Astron. Soc.* **306**, 89–94 (1999).
5. Thielemann, F.-K. *et al. Astrophys. J.* **460**, 408–436 (1996).
6. Woosley, S. & Weaver, T. *Astrophys. J. Suppl.* **101**, 181–235 (1995).

Genomics

The millennium flies in

Kenneth C. Burtis and R. Scott Hawley

Almost a century has passed since the first steps in the genetic analysis of the fly *Drosophila melanogaster* were taken by W. E. Castle at Harvard University. Another major milestone will be reached in the next few months as the completed sequence of the *Drosophila* genome is released, reflecting the combined efforts of the Berkeley and European *Drosophila* Genome Projects and Celera Genomics. Papers by Ashburner *et al.*¹ and Spradling *et al.*² in this month's *Genetics* preview the huge amount of work that will be involved in correlating the primary DNA sequence with genetic function. This link is essential if we are to bring full biological relevance to the flood of raw data produced by this and other projects to sequence the genomes of 'model' organisms.

The paper by Ashburner *et al.*¹ presents the annotation of 2.9 Mb of DNA surrounding the alcohol dehydrogenase (*Adh*) gene, one of the most thoroughly characterized regions of the fly genome. The companion paper by Spradling *et al.*² summarizes the progress in ongoing attempts to produce a comprehensive set of gene disruptions in the fly. Spradling and his collaborators have accumulated and characterized a collection of transposon-induced mutations in roughly a quarter of all the genes that can readily be defined by mutation (lethals, steriles and visibles). The authors also present a plan for sequence-based studies that should eventu-

ally allow insertional disruption of almost all *Drosophila* genes, irrespective of how (and whether) these changes manifest themselves in the mutant. Finally, Spradling *et al.* describe roughly 1,000 disrupted genes—the largest collection of genes for which both molecular and genetic data are available in a model, multicellular, eukaryotic system.

Ashburner *et al.* report the culmination of work by a vast number of researchers who contributed the mutants, chromosome aberrations and cloned sequences. Of the 218 protein-coding genes identified by sequence analysis, about one-third (73) have been previously defined. Ashburner and colleagues have now mapped 658 aberration breakpoints in the *Adh* region, providing critical landmarks for gene localization. The authors have also positioned 181 places where a movable stretch of DNA called the P element is independently inserted in the genome by a process known as transposition. Their sequence analysis provides an insight into issues ranging from gene density to the frequency and size of gene duplications. It also provides the last nail in the coffin for the view that, in the *Drosophila* polytene chromosome (a large chromosome with a characteristic 'banded' appearance), one band corresponds to one gene. This view was promulgated by Calvin Bridges, the first practitioner of functional genomics in flies or any other organism, over half a century ago.

But Ashburner *et al.* now find 218 protein-coding genes and 11 genes encoding transfer RNA in a region comprising only 69 bands on the polytene chromosome.

Of the 218 protein-coding genes in this 2.9-Mb region, 44% are represented in the existing collection of *Drosophila* expressed-sequence tags (ESTs; probes of short DNA sequences for particular genes), and about 70% encode proteins with similar ('conserved') sequences to those found in other organisms. Remarkably, those genes that have been identified previously by mutants with phenotypes (or manifestations) such as lethality, sterility or changes in appearance were five to 18 times (depending on the probability threshold used to define conservation) more likely to be conserved between organisms than those sequences without an identified function.

One could argue that the phenotypes of mutationally undefined genes simply remain to be discovered. However, a large number of saturation-level mutagenesis experiments have been done on the *Adh* region over the past few decades, so it is very unlikely that many genes for which mutants produce obvious phenotypes remain to be defined. Rather, this large collection of data supports the hypothesis that genes with essential functions tend not to be organism-specific, and that the sequences encoding non-essential functions are less likely to be conserved. How, then, are the functions—if any—of these non-essential genes to be defined?

As recognized by those who study yeast, a crucial complement to the full genomic sequence is a comprehensive set of gene knockouts. Although site-directed mutagenesis is not possible in flies, Spradling *et al.*² show that, by transposition of the P element, it will be possible to insertional inactivate most genes in the fly genome. These authors show the existence of sites with intermediate levels of bias for P-element insertion—sites that confounded previous attempts to estimate the number of independent insertions required to ensure that each gene is defined by at least one insertion. Conservative estimates suggest that Spradling and collaborators might need to screen more than 150,000 lines to obtain insertions in 87% of all genes using the initial methodology. Fortunately, as the authors themselves point out, with the complete genomic sequence, high-throughput sequence-based molecular approaches can be used in a second, comprehensive round of transposon-mediated mutagenesis. Identification of insertional disruptions by flanking sequence (rather than by phenotype) means that all genes will be potential targets. Moreover, using recently developed transposons³, it should be possible to generate informative gain-of-expression characteristics for genes (such as those with redundant or overlapping functions) that would not otherwise be detectable.

The effort required to annotate and characterize the *Drosophila* genome will be proportional to the genome's size and the number of genes. Current estimates, from extrapolation of partial sequence data, give a total size of 140–150 Mb. Predicting the number of genes is a greater challenge — one that is at the heart of the annotation studies. Depending on the assumptions used, extrapolations based on the relative number of polytene bands, vital genes, predicted genes and DNA content in the *Adh* region lead Ashburner *et al.* to estimates ranging from 8,600 to almost 17,000 genes, bracketing a previous estimate⁴ of 12,000 genes. The *Adh* region was, in fact, the subject of a recent annotation competition⁵. Most of the participants predicted between 20 and 30 more protein-coding genes than the 218 found by Ashburner *et al.*, highlighting the importance of functional studies in validating the predictions from bioinformatic approaches. But even if *Drosophila* turns out to have 10% more genes than the current predicted total, it will still probably have fewer than the 19,000 genes in the nematode worm *Caenorhabditis elegans*. This is surprising, given that flies are more complex than worms at many levels. To understand this obvious case of 'less is more', we will need to unravel the functional genomics of both organisms.

The community of *Drosophila* researchers is well positioned to do the functional studies that will be required to meet the challenges presented by release of the genomic sequence. The impressively large number of genes with disparate functions that map to the *Adh* interval reflects the extraordinary number and diversity of laboratories that use *Drosophila* as an experimental system. The *Drosophila* community extends well beyond those who focus on developmental genetics, and includes researchers whose interests and technical abilities embrace such diverse disciplines as biochemistry, cell biology, population studies and evolution. With the complete *Drosophila* gene list and gene knockouts, we can expect that this little bug, whose biology began genetics in this century, will be many times more useful in the next. ■

Kenneth C. Burtis and R. Scott Hawley are in the Department of Genetics, Section of Molecular and Cellular Biology, University of California, One Shields Avenue, Davis, California 95616, USA. e-mails: shawley@netcom.com kcburtis@ucdavis.edu

1. Ashburner, M. *et al.* *Genetics* **153**, 179–219 (1999).
2. Spradling, A. C. *et al.* *Genetics* **153**, 135–177 (1999).
3. Rorth, P. *et al.* *Development* **125**, 1049–1057 (1998).
4. Miklos, G. L. G. & Rubin, G. M. *Cell* **86**, 521–529 (1996).
5. Abbott, A. *Nature* **400**, 699 (1999).

Apoptosis

Condensed matter in cell death

Naoufal Zamzami and Guido Kroemer

Since the nineteenth century, anatomists and pathologists have observed strikingly condensed nuclei with clumped, condensed chromatin (DNA complexed with proteins) as a sign of cell death. This phenomenon, termed 'pyknosis', is often accompanied by 'karyorrhexis' — fragmentation of the nucleus. Today, at the twentieth *fin de siècle*, these terms lose some of their mystery. For on page 168 of this issue, Sahara *et al.*¹ report the molecular characterization of a hitherto unknown factor, Acinus (for 'apoptotic chromatin condensation inducer in the nucleus'). This factor seems to be indispensable for nuclear pyknosis, at least in some experimental settings.

Nuclear pyknosis and karyorrhexis are near-to-definitive hallmarks of programmed cell death (apoptosis). In teleological terms, apoptosis is a type of death designed specifically to disintegrate cells in an ordered fashion, avoiding pathogenic spillover of the cell's soluble components into tissues. This is done by maintaining the barrier function of the plasma membrane. Nevertheless, there are subtle changes in this membrane, causing neighbouring cells to engulf the dying

cells. So, the doomed cell is degraded within the limits of a vesicle — the phagolysosome — of another healthy cell.

To facilitate such engulfment, apoptotic cells reduce their volume. They pump out ions (mainly K⁺) and contract their reorganized cytoskeleton, which forms a sort of cage around the nucleus. Overall shrinkage is usually accompanied by nuclear pyknosis. Cells may also detach parts of their cytoplasm, which sometimes includes highly condensed fragments of the karyorrhectic nucleus. The size and membrane characteristics of these 'apoptotic bodies' makes them very palatable to neighbouring healthy cells. The dying cells also activate catabolic enzymes that ensure digestion of critical cellular components from the inside (as

opposed to enzymes in the phagolysosome, which have to act from the outside). Such catabolic hydrolases include a class of specific protein-cleaving enzymes (caspases), as well as DNA-digesting enzymes (DNases), both of which participate directly or indirectly in nuclear pyknosis.

Which factors are responsible for chromatin condensation and pyknosis? In most instances, addition of caspase inhibitors prevents pyknosis. So, caspases may act upstream of other effectors, or they may act directly on nuclear proteins to dismantle the nuclear envelope and facilitate the condensation and digestion of chromatin. Sahara *et al.*¹ now suggest that caspase-3 cleaves Acinus, which is the precursor of a chromatin-condensation factor. Cleavage of Acinus at a specific aspartate residue (Asp 1093) by caspase-3 is necessary — but not sufficient — to activate the DNA-condensing activity of Acinus. For full activation, an additional (unknown) protease cleaving at serine 987 (Ser 987) has to intervene. Only the combined action of both proteases generates the mature Ser 987 to Asp 1053 fragment, which, when added to purified nuclei in the presence of unidentified nuclear-import factors, causes chromatin condensation *in vitro*.

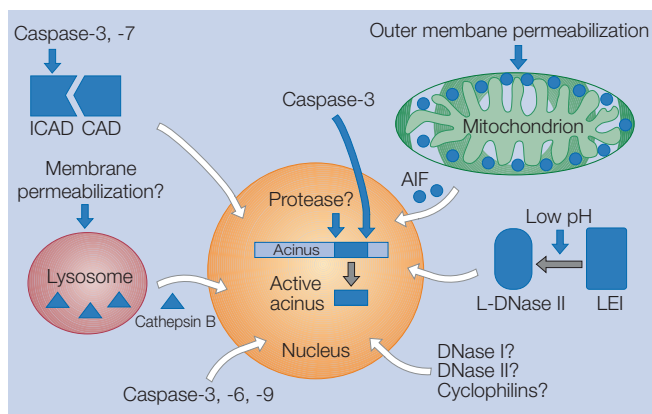
Although Acinus is not the only factor to condense chromatin during apoptosis (Table 1), Sahara and colleagues show that it has a unique property — it does so in the absence of DNase activity. Another chromatin-condensation factor, the caspase-activated DNase (CAD), for example, uses its DNase activity to cleave chromatin at the boundaries between nucleosomes (the histone–DNA complexes that make up chromatin). In this way, CAD generates stretches of DNA about 200 base pairs (or multiples thereof) long². The CAD protein is also activated by caspases, albeit indirectly. When caspase-3 or caspase-7 digests its inhibitor (ICAD), CAD is released from suppression and translocates to the nucleus. There it interacts with histone H1 and high-mobility-group 2 proteins, and exerts its DNase and chromatin-condensation activities². So, as a general rule, caspases activate non-caspase effectors that participate in nuclear apoptosis³ (Fig. 1, overleaf).

Does chromatin condensation, then, always require a caspase-dependent step? The answer is clearly no. For instance, partial or complete chromatin condensation is observed when the tumour-suppressor gene PML is overexpressed⁴, or when the HIV-1 co-receptor CXCR4 is crosslinked⁵ — even

Table 1 Nuclear effects of apoptosis-promoting molecules

	Acinus	CAD	AIF	L-DNase II	Cath B	DNase I	DNase II	Cyclophilin	Caspases
Type of chromatin condensation	Karyo-rhexis	Karyo-rhexis	Peri-pher	Pyk-nosis	Peri-pher	Pyk-nosis	Pyk-nosis	?	None
Type of DNA fragmentation	None	Oligos	≥50 kb	Oligos	Oligos	Oligos	≥50 kb	None	
Proteolysis	?	None	None	None	?	None	None	None	

Figure 1 Apoptotic effectors that act on the nucleus. Disruption of intracellular compartmentalization (which is well established for mitochondria but putative for lysosomes), or pH-driven conformational change, culminates in the nuclear import of effector proteins — such as caspase-activated DNase (CAD), apoptosis-inducing factor (AIF), cathepsin B, L-DNase II — or in the activation of Acinus, which is already present in the nucleus. Sahara *et al.*¹ report that cleavage of Acinus by caspase-3 and an unknown protease generates an active fragment that can cause chromatin condensation, despite lacking DNase activity. It is not clear which pathways co-activate and/or require Acinus for chromatin condensation. Other proteins with DNase activity, which are thought to be involved in apoptotic digestion of chromatin, are also listed.



in the absence of caspase activation. Similarly, inactivation of the caspase-3 gene does not prevent chromatin condensation. Rather, it modifies the pattern of the process, or retards it according to different cell types or stimuli^{6,7}.

One of the proteins responsible for caspase-independent chromatin condensation has, in fact, been identified. Known as the apoptosis-inducing factor (AIF), it is a flavoprotein that is normally confined to the space between the outer and inner mitochondrial membranes⁸. Once apoptosis is induced, AIF translocates from the mitochondrion to the nucleus, where it causes partial chromatin condensation in the periphery of the nucleus⁸. This pattern of condensation is clearly distinct from those induced by CAD (ref. 3) or Acinus¹. AIF causes degradation of DNA into fragments greater than around 50 kb in length⁸ — a large-scale DNA fragmentation that precedes the more advanced internucleosomal degradation.

Another chromatin-condensation factor, which translocates from the cytoplasm to the nucleus, is L-DNase II (Fig. 1). This protein is derived from a serpin-like protease inhibitor, leukocyte elastase inhibitor, through a post-translational modification that may be triggered by cytosolic acidification⁹, a metabolic change that often accompanies apoptosis. When added to purified nuclei, the L-DNase II causes a marked chromatin condensation and chops the chromatin into nucleosome-sized fragments⁹. Yet another protein that might contribute to chromatin condensation and internucleosomal DNA fragmentation is cathepsin B. This protein could be activated on its release from lysosomes of the apoptotic cell¹⁰. Alternatively, cathepsins might act as degradative enzymes in the phagolysosome.

Why are there so many chromatin-condensation factors? One possibility is

sequential action. For example, AIF may act before the caspase-dependent chromatin-condensation factors⁸. Another idea is complementarity, which is evident from the many different effects of the distinct apoptosis-promoting molecules that act on the nucleus (Table 1). Finally, redundancy is suggested because apoptotic extracts can contain CAD and other chromatin-condensation factors³, including Acinus¹. Redundancy is underlined by the observation that, in Jurkat cells, overexpression of a caspase-3-resistant ICAD mutant prevents CAD-mediated DNA fragmentation, yet fails to abolish (Acinus-mediated?) chromatin condensation¹¹. Some chromatin-condensation factors such as CAD are expressed in a strictly tissue-specific fashion¹², indicating that chromatin condensation may result from distinct molecular processes in different cell types. This means that, although they are morphologically uniform, pyknosis and karyorrhexis may result from highly specialized processes. Part of the mystery remains to be pierced.

Naoufal Zamzami and Guido Kroemer are in the Apoptosis, Cancer and Immunity Laboratory associated with the National League against Cancer, CNRS-ERS1984, 19 rue Guy Môquet, F-94801 Villejuif, France.

e-mail: kroemer@infobiogen.fr

- Sahara, S. *et al.* *Nature* **401**, 168–173 (1999).
- Enari, M. *et al.* *Nature* **391**, 43–50 (1998).
- Samejima, K. *et al.* *J. Cell Biol.* **143**, 225–239 (1998).
- Quignon, F. *et al.* *Nature Genet.* **20**, 259–265 (1998).
- Berndt, C., Möpps, B., Angermüller, S., Gierschik, P. & Krammer, P. H. *Proc. Natl Acad. Sci. USA* **95**, 12556–12561 (1998).
- Woo, M. *et al.* *Genes Dev.* **12**, 806–819 (1998).
- Zheng, T. S. *et al.* *Proc. Natl Acad. Sci. USA* **95**, 13618–13623 (1998).
- Susin, S. A. *et al.* *Nature* **397**, 441–446 (1999).
- Torriglia, A. *et al.* *Mol. Cell. Biol.* **18**, 3612–3619 (1998).
- Vancompernelle, K. *et al.* *FEBS Lett.* **6**, 150–158 (1998).
- Sakahira, H., Enari, M., Ohsawa, Y., Uchiyama, Y. & Nagata, S. *Curr. Biol.* **9**, 543–546 (1999).
- Mukae, N. *et al.* *Proc. Natl Acad. Sci. USA* **95**, 9123–9128 (1998).

Daedalus

Weed insulation

Last week Daedalus proposed a ship coated with ice. Not only would it have the lowest possible skin-friction; fouling organisms could never grow on its hull. He now doubts this latter assertion. No marine plant is known to anchor itself to ice. But many specialized bacteria can live in ice, and tundra lichens can survive in an environment dominated by it. So perhaps a seaweed exists, or can be bred, to flourish on an icy surface.

Many seaweeds anchor themselves to solid surfaces, both naval and natural. They do so, not to draw nourishment from the surface, but to contact a large volume of water as it sweeps past their anchorage. A seaweed that could fasten itself to ice would gain the same benefit. DREADCO biologists are now trying to create one.

The trouble, of course, is that natural ice is usually melting. No plant can stick to its surface; it needs to carve out a cavity. Daedalus recalls that Arctic fish, like many other cold-adapted species, generate high internal concentrations of special anti-freeze compounds. If the genes for such a compound could be transferred to seaweed, and the plant could learn to exude it from its anchoring foot, it could melt out a secure root-cavity in the ice. As fast as the ice melted, it would deepen and renew its cavity. It could spread as a permanent coating on the submerged surface of an ice shelf, even colonizing the underneath. Enough light would penetrate the ice to sustain photosynthesis.

This challenging biological project has a bold ecological purpose — to reduce the melt-rate of the polar ice. During the Second World War, an artificial iceberg was proposed as an aircraft-carrier. To reduce its melt-rate, it was to be made from a dilute suspension of wood-pulp in water. This would give it a furry surface, which would be an excellent thermal insulator. Similarly, the Arctic and Antarctic polar ice shelves could be insulated by seeding them with Daedalus's ice-weed.

A menace of global warming would thus be countered. Released in the polar oceans, ice-weed would spread luxuriantly under the polar ice shelves and submarine glaciers, reducing their rate of melting. Their contribution to the rising sea-level would be cut right back. They would also release fewer free-floating icebergs to menace the world's shipping lanes. The bergs that were released would, of course, be thoroughly weed-insulated, and would last much longer. They might even be captured and towed into tropical ports for use as sources of fresh water.

David Jones

Tropical tree gene flow and seed dispersal

Deforestation affects the genetic structure of the surviving forest fragments.

In tropical forests, trees provide habitats and environmental conditions that support thousands of species. However, deforestation creates a mosaic landscape of cleared areas and forest fragments, which become the source of future tree populations. Fragmentation changes the movement of pollen and seed dispersal, modifying the gene flow and altering historical patterns of genetic subdivision. Paternity studies in tropical figs¹ and trees² have shown that pollen is dispersed over long distances, maintaining gene flow among widely spaced forest fragments, but gene movement by seed dispersal has not been studied in tropical trees. I have estimated chloroplast genome subdivision in the Amazonian canopy tree *Corythophora alta*, and here I show that seed dispersal is limited, with forest fragments as large as ten hectares being founded by a single maternal lineage.

Because trees are generally long-lived, the population-genetic effects of forest fragmentation are difficult to observe directly. Studies of gene flow provide an indirect means of gauging these effects and comparing the genetic structure in intact and fragmented forest. We can use the dispersal distances of pollen and seeds to estimate the size of a genetic 'neighbourhood' or the breeding area of a population. Paternal genes disperse through both pollen and seed, whereas maternal genes disperse only through seed. Total gene dispersal $\sigma^2 = (\sigma_p^2/2) + \sigma_s^2$, where σ_p^2 and σ_s^2 are the variances in pollen and seed movement, respectively, and the factor of 1/2 is required because pollen is haploid and we should omit the contribution of maternal gametes which do not disperse³. Seed-dispersal variance therefore contributes twice as much as pollen-dispersal variance to the size of genetic neighbourhoods.

Tissues were sampled from 162 mature trees (of at least 10 cm diameter at breast height) in four forest fragments and three sites of continuous forest at the Biological Dynamics of Forest Fragments Project near

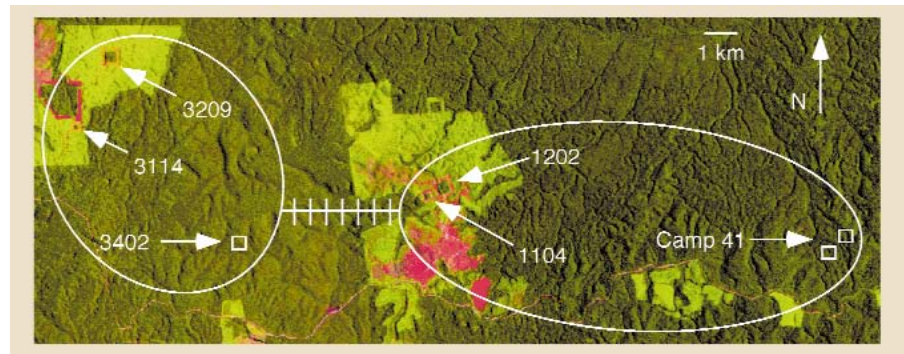


Figure 1 A 1995 Landsat image of the *C. alta* populations showing patterns of chloroplast (cp) DNA variation. Squares indicate continuous forest populations. Ellipses group populations with identical cpDNA haplotypes and the connecting line indicates seven haplotype differences between groups. Two trees with unique psbB-psbF haplotypes in population 3402 are not shown. Dark green, continuous forest; light green, second growth; pink, pasture.

Manaus, Brazil (Fig. 1). The forest fragments were created in the early 1980s but mature trees established before forest clearance, as judged from growth rates for tree species of the family Lecythidaceae⁴. The sites are located within relatively homogeneous forest and are not separated by physical or ecological barriers to seed dispersal.

Chloroplast (cp) DNA is maternally inherited in most angiosperms⁵ and disperses only through seeds, so the spatial scale of cpDNA genetic differences among populations is a direct estimate of previous seed dispersal that led to trees being established⁶. Insertion or deletion polymorphisms were identified by sequencing five cpDNA regions^{7,8}. Individual cpDNA haplotypes for seven polymorphisms were determined by allele-specific polymerase chain reaction (PCR). DNA sequence from random individuals verified that those with identical PCR haplotypes have identical cpDNA sequences. Haplotype frequencies among forest sites (Table 1) indicate almost complete cpDNA subdivision (the fixation index among populations, $F_{ST} = 0.992 \pm 0.042$; calculated according to ref. 9) among forest sites less than 10 km apart (Fig. 1). Forest fragments of 1 and 10 hectares all contain trees with a single cpDNA haplotype, indicating that

they had a single maternal founder.

The pattern of cpDNA subdivision is consistent with historical seed dispersal being limited to small areas of continuous tropical forest. However, the seven populations are resolved into only two haplotype groups, so the extent of a seed-dispersal neighbourhood has not been estimated. Furthermore, selection can increase population subdivision in non-recombining organelle genomes¹⁰, although there are no obvious differences in forest composition over this small spatial scale that could produce localized selection for cpDNA variants. I am now collecting data from adjacent populations and testing whether evolution in the chloroplast genome is consistent with a neutral model of substitution.

My results show that restricted seed-mediated gene flow has led to sharp genetic differentiation of adjacent populations. They indicate that small forest plots may exchange seeds infrequently, limiting the size of genetic neighbourhoods. Studies of gene flow in tropical trees have focused mostly on pollen dispersal, rather than seed dispersal, which may be more limited than pollen dispersal and is as likely to be altered by forest disturbance. Because gene flow through seeds is responsible for two-thirds of the total genetic-neighbourhood size, it is essential for estimates of the size of tropical-tree breeding populations. Gene flow from both seed and pollen dispersal will be required to predict fragmentation-induced genetic changes in tropical forest trees.

Matthew B. Hamilton

Georgetown University, Department of Biology, Reiss Sciences 406, Washington DC 20057, USA and Biological Dynamics of Forest Fragments Project, National Institute for Research in the Amazon, CP 478, Manaus, AM 69011-970, Brazil
e-mail:hamiltmb@gusun.georgetown.edu

Table 1 Frequency of chloroplast genome insertion haplotypes for *C. alta*

Population	Size (ha)	Number	1	2	3	4	5	6	7
1202	10	61	1.0	1.0	0.0	1.0	0.0	0.0	1.0
1104	1	1	1.0	1.0	0.0	1.0	0.0	0.0	1.0
41-1	10	16	1.0	1.0	0.0	1.0	0.0	0.0	1.0
41-2	10	20	1.0	1.0	0.0	1.0	0.0	0.0	0.0
3209	10	27	0.0	0.0	1.0	0.0	1.0	1.0	1.0
3114	1	8	0.0	0.0	1.0	0.0	1.0	1.0	0.0
3402	10	29	0.0	0.0	1.0	0.07	1.0	1.0	0.0

The cpDNA sites are: 1, trnL intron; 2, trnH-psbA 1; 3, trnH-psbA 2; 4, psbB-psbF; 5, rpl20-5' rps12; 6, trnS-trnG 172; and 7, trnS-trnG 520. Primers are described in ref. 8 for site 1 and in ref. 7 for all other sites.

1. Chase, M. R., Moller, C., Kesseli, R. & Bawa, K. S. *Nature* **383**, 398–399 (1996).
2. Nason, J. D., Allen Herre, E. & Hamrick, J. L. *Nature* **391**, 685–687 (1998).
3. Crawford, T. J. *Heredity* **52**, 273–283 (1984).
4. Chambers, J. Q., Higuchi, N. & Schimel, J. P. *Nature* **391**, 135–136 (1998).

5. Reboud, X. & Zeyl, C. *Heredity* **72**, 132–140 (1994).
6. McCauley, D. E. *Trends Ecol. Evol.* **10**, 198–202 (1995).
7. Hamilton, M. B. *Mol. Ecol.* **8**, 521–522 (1999).
8. Taberlet, P. *et al.* *Plant Mol. Biol.* **17**, 1105–1109 (1991).
9. Weir, B. S. *Genetic Data Analysis II* (Sinauer, Sunderland, Massachusetts, 1996).
10. Rand, D. M. *Conserv. Biol.* **10**, 665–671 (1996).

Internet

Diameter of the World-Wide Web

Despite its increasing role in communication, the World-Wide Web remains uncontrolled: any individual or institution can create a website with any number of documents and links. This unregulated growth leads to a huge and complex web, which becomes a large directed graph whose vertices are documents and whose edges are links (URLs) that point from one document to another. The topology of this graph determines the web's connectivity and consequently how effectively we can locate information on it. But its enormous size (estimated to be at least 8×10^8 documents¹) and the continual changing of documents and links make it impossible to catalogue all the vertices and edges.

The extent of the challenge in obtaining a complete topological map of the web is illustrated by the limitations of the commercial search engines: Northern Light, the search engine with the largest coverage, is estimated to index only 38% of the web¹. Although much work has been done to map and characterize the Internet's infrastructure², little is known about what really matters in the search for information — the topology of the web. Here we take a step towards filling this gap: we have used local connectivity measurements to construct a topological model of the World-Wide Web, which has enabled us to explore and characterize its large-scale properties.

To determine the local connectivity of the web, we constructed a robot that adds to its database all URLs found on a document and recursively follows these to retrieve the related documents and URLs. We used the data collected to determine the probabilities $P_{out}(k)$ and $P_{in}(k)$ that a document has k outgoing and incoming links, respectively. We find that both $P_{out}(k)$ and $P_{in}(k)$ follow a power law over several orders of magnitude, remarkably different not only from the Poisson distribution predicted by the classical theory of random graphs^{3,4}, but also from the bounded distribution found in models of random networks⁵.

The power-law tail indicates that the probability of finding documents with a large number of links is significant, as the network connectivity is dominated by highly connected web pages. Similarly, for

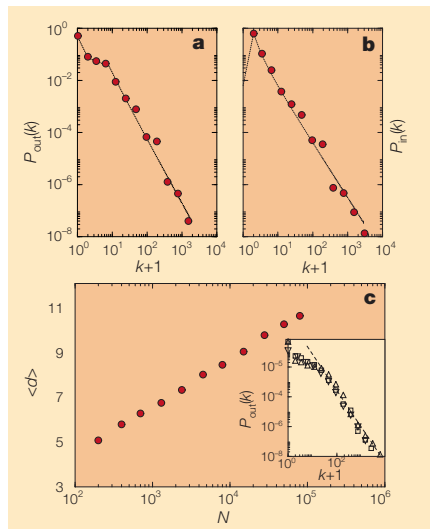


Figure 1 Distribution of links on the World-Wide Web. **a**, Outgoing links (URLs found on an HTML document); **b**, incoming links (URLs pointing to a certain HTML document). Data were obtained from the complete map of the nd.edu domain, which contains 325,729 documents and 1,469,680 links. Dotted lines represent analytical fits used as input distributions in constructing the topological model of the web; the tail of the distributions follows $P(k) \approx k^{-\gamma}$, with $\gamma_{out} = 2.45$ and $\gamma_{in} = 2.1$. **c**, Average of the shortest path between two documents as a function of system size, as predicted by the model. To check the validity of our predictions, we determined d for documents in the domain nd.edu. The measured $\langle d_{nd.edu} \rangle = 11.2$ agrees well with the prediction $\langle d_{8 \times 10^8} \rangle = 11.6$ obtained from our model. To show that the power-law tail of $P(k)$ is a universal feature of the web, the inset shows $P_{out}(k)$ obtained by starting from whitehouse.gov (squares), yahoo.com (triangles) and snu.ac.kr (inverted triangles). The slope of the dashed line is $\gamma_{out} = 2.45$, as obtained from nd.edu in **a**.

incoming links, the probability of finding very popular addresses, to which a large number of other documents point, is non-negligible, an indication of the flocking nature of the web. Furthermore, while the owner of each web page has complete freedom in choosing the number of links on a document and the addresses to which they point, the overall system obeys scaling laws characteristic only of highly interactive self-organized systems and critical phenomena⁶.

To investigate the connectivity and the large-scale topological properties of the web, we constructed a directed random graph consisting of N vertices, assigning to each vertex k outgoing (or incoming) links, such that k is drawn from the power-law distribution of Fig. 1a,b. To achieve this, we randomly selected a vertex i and increased its outgoing (or incoming) connectivity to $k_i + 1$ if the total number of vertices with

$k_i + 1$ outgoing (or incoming) links is less than $NP_{out}(k_i + 1)$ (or $NP_{in}(k_i + 1)$).

A particularly important quantity in a search process is the shortest path between two documents, d , defined as the smallest number of URL links that must be followed to navigate from one document to the other. We find that the average of d over all pairs of vertices is $\langle d \rangle = 0.35 + 2.06 \log(N)$ (Fig. 1c), indicating that the web forms a small-world network^{5,7}, which characterizes social or biological systems. For $N = 8 \times 10^8$, $\langle d_{web} \rangle = 18.59$; that is, two randomly chosen documents on the web are on average 19 clicks away from each other.

For a given N , d follows a gaussian distribution so $\langle d \rangle$ can be interpreted as the diameter of the web, a measure of the shortest distance between any two points in the system. Despite its huge size, our results indicate that the web is a highly connected graph with an average diameter of only 19 links. The logarithmic dependence of $\langle d \rangle$ on N is important to the future potential of the web: we find that the expected 1,000% increase in the size of the web over the next few years will change $\langle d \rangle$ very little, from 19 to only 21.

The relatively small value of $\langle d \rangle$ indicates that an intelligent agent, who can interpret the links and follow only the relevant one, can find the desired information quickly by navigating the web. But this is not the case for a robot that locates the information based on matching strings. We find that such a robot, aiming to identify a document at distance $\langle d \rangle$, needs to search $M(\langle d \rangle) \approx 0.53 \times N^{0.92}$ documents, which, with $N = 8 \times 10^8$, leads to $M = 8 \times 10^7$, or 10% of the whole web. This indicates that robots cannot benefit from the highly connected nature of the web, their only successful strategy being to index as much of the web as possible.

The scale-free nature of the link distributions indicates that collective phenomena play a previously unsuspected role in the development of the web⁸, forcing us to look beyond the traditional random graph models^{3–5,7}. A better understanding of the web's topology, aided by modelling efforts, is crucial in developing search algorithms or designing strategies for making information widely accessible on the World-Wide Web. Fortunately, the surprisingly small diameter of the web means that all that information is just a few clicks away.

Réka Albert, Hawoong Jeong, Albert-László Barabási

Department of Physics, University of Notre Dame, Notre Dame, Indiana 46556, USA
e-mail: alb@nd.edu

1. Lawrence, S. & Giles, C. L. *Nature* **400**, 107–109 (1999).
2. Claffy, K., Monk, T. E. & McRobb, D. Internet tomography. *Nature* [online] <http://helix.nature.com/webmatters/tomog/tomog.html> (1999).
3. Erdős, P. & Rényi, A. *Publ. Math. Inst. Hung. Acad. Sci.* **5**, 17–61 (1960).
4. Bollobás, B. *Random Graphs* (Academic, London, 1985).

5. Watts, D. J. & Strogatz, S. H. *Nature* **393**, 440–442 (1998).
6. Bunde, A. & Havlin, S. *Fractals in Science* (Springer, Berlin, 1994).
7. Barthélemy, M. & Amaral, L. A. N. *Phys. Rev. Lett.* **82**, 3180–3183 (1999).
8. Barabási, A.-L., Albert, R. & Jeong, H. <<http://www.nd.edu/~networks>>.

Internet

Growth dynamics of the World-Wide Web

The exponential growth of the World-Wide Web has transformed it into an ecology of knowledge in which highly diverse information is linked in an extremely complex and arbitrary manner. But even so, as we show here, there is order hidden in the web. We find that web pages are distributed among sites according to a universal power law: many sites have only a few pages, whereas very few sites have hundreds of thousands of pages. This universal distribution can be explained by using a simple stochastic dynamical growth model.

The existence of a power law in the growth of the web not only implies the lack of any length scale for the web, but also allows the expected number of sites of any given size to be determined without exhaustively crawling the web. The distribution of site sizes for crawls by Alexa and Infoseek is shown in Fig. 1. Both data sets display a power law over several orders of magnitude, so on a log–log scale the distribution of the number of pages per site appears as a straight line. This distribution should not be confused with Zipf's like distributions^{1,2}, where a power law arises from rank ordering the variables³.

In order to describe the growth process underlying this distribution⁴, we assume

that the day-to-day fluctuations in site size are proportional to the size of the site. One would not be surprised to find that a site with a million pages has lost or gained a few hundred pages on any given day. On the other hand, finding an additional hundred pages on a site with just ten pages within a day would be unusual. So we assume that the number of pages on the site, n , on a given day, is equal to the number of pages on that site on the previous day plus or minus a random fraction of n .

If a set of sites is allowed to grow with the same average growth rate but with individual random daily fluctuations in the number of pages added, their sizes will be distributed log-normally after a sufficiently long period of time⁵. A log-normal distribution gives high probability to small sizes and small, but significant, probability to very large sizes. But although it is skewed and has a long tail, the log-normal distribution is not a power-law one.

Two additional factors that determine the growth of the web need to be considered: sites appear at different times and grow at different rates. The number of web sites has been growing exponentially since its inception, which means that there are many more young sites than old ones. Once the age of the site is factored in to the multiplicative growth process, $P(n)$, the probability of finding a site of size n , is a power law, that is, it is proportional to $n^{-\beta}$. Similarly, considering sites with a wide range of distributions in growth rates yields the same result: a power-law distribution in site size. The simple assumption of stochastic multiplicative growth, combined with the fact that sites appear at different times and/or grow at different rates, therefore leads to an explanation of the observed power-law behaviour.

The existence of this universal power law, which is yet another example of the strong regularities^{6,7} revealed by studies of the web, also has practical consequences. The expected number of sites of any arbitrary size can be estimated, even if a site of that size has not yet been observed. This can be achieved by extrapolating the power law to any large n ; for example, $P(n_2) = P(n_1) \times (n_2/n_1)^{-\beta}$. The expected number of sites of size n_2 in a crawl of N sites would be $NP(n_2)$. For instance, from the Alexa data we can infer that, if data were collected from 250,000 sites, the probability of finding a site with a million pages would be 10^{-4} . This information is not readily available from the crawl alone, as it stops at 10^5 pages per site.

Bernardo A. Huberman, Lada A. Adamic
Xerox Palo Alto Research Center,
3333 Coyote Hill,
Palo Alto, California 94304, USA
e-mail: ladamic@parc.xerox.com

1. Zipf, G. K. *Human Behavior and the Principle of Least Effort* (Addison-Wesley, Cambridge, Massachusetts, 1949).
2. Mantegna, R. N. *et al. Phys. Rev. E* **52**, 2939–2950 (1995).

3. Gunther, R. *et al. Int. J. Theor. Phys.* **35**, 395–417 (1996).
4. <http://www.parc.xerox.com/ica/www/growth.html>
5. Crow, E. L. & Shimizu, K. *Lognormal Distributions: Theory and Applications* (Dekker, New York, 1988).
6. Huberman, B. A., Pioroli, P., Pitkow, J. & Lukose, R. M. *Science* **280**, 95–97 (1998).
7. Huberman, B. A. & Lukose, R. M. *Science* **277**, 535–538 (1997).

Genome evolution

Global methylation in eutherian hybrids

O'Neill *et al.* propose that epigenetic processes help to drive karyotypic evolution in marsupials¹. Here we present evidence that global methylation patterns do not undergo dramatic changes in interspecific hybrids among three orders of placental mammals, indicating that the mechanisms underlying genome evolution may be different in placental mammals and marsupials.

Interspecific hybridization in mammals frequently results in male sterility², abnormal growth³ and placental dysplasia^{3–5}, which together may cause post-meiotic reproductive isolation. It has been proposed that incompatibility between rapidly evolving genes that interact normally in the intraspecific context⁶ and genomic rearrangements⁷ may explain interspecific hybrid defects.

O'Neill *et al.* have given a striking example for the latter mechanism in an interspecific hybrid of the marsupials *Macropus eugenii* and *M. bicolor*¹. This first-generation (F_1) hybrid exhibited genome-wide demethylation, retrotransposon amplification and centromere expansion on the autosomes derived from *M. eugenii*. Undermethylation of F_1 genomes compared with those of the parental species was also seen in two hybrids of other species within the genus *Petrogale*¹. These findings were taken to indicate that retrotransposon amplification and chromosome expansion secondary to genome-wide undermethylation could be a frequent phenomenon in mammalian hybrids, leading to rapid karyotypic evolution and finally to reproductive isolation¹.

We have analysed genome-wide methylation in interspecific hybrids in the placental mammalian families of three orders, Equidae (Perissodactyla), Muridae (Rodentia) and Camelidae (Artiodactyla), by following the digestion of genomic DNA with the methylation-sensitive and methylation-insensitive enzymes *HpaII* and *MspI*, respectively, and Southern blotting the digest. This analysis included hybrids between horse and donkey, three species of mouse (*Mus musculus*, *Mus spretus* and *Mus macedonicus*), and llama (*Lama glama*) and dromedary (*Camelus dromedarius*). This analysis gave no indication for any changes in genome-wide methylation in any of the F_1 hybrids when compared with parental animals (Fig. 1).

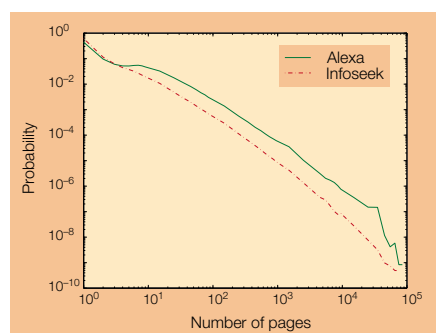


Figure 1 Log–log plot of the distribution of pages in sites for Alexa and Infoseek crawls, which covered 259,794 and 525,882 sites, respectively. There is a drop-off at approximately 10^5 pages because server limitations mean that search engines do not systematically collect more pages per site than this. A linear regression on the variables $\log(\text{number of sites})$ and $\log(\text{number of pages})$ yielded [1.647, 1.853] as the 95% confidence interval for the exponent β in the Alexa crawl, and [1.775, 1.909] for the Infoseek crawl. These estimates for the power-law slope are consistent across the two data sets and with the model, which predicts that β is greater than 1.

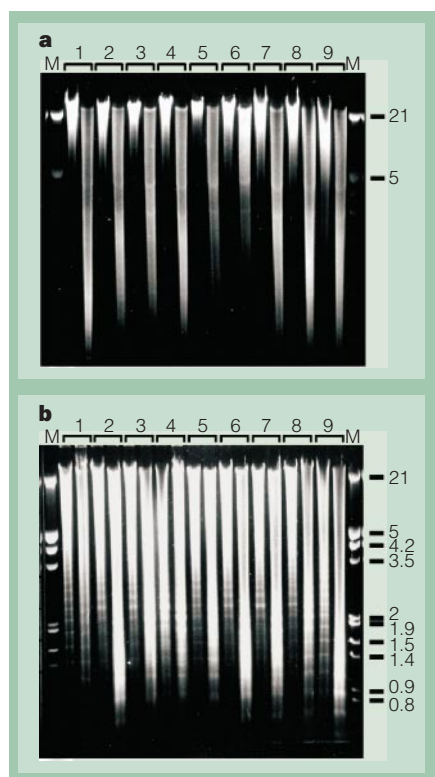


Figure 1 Genome methylation analysis in interspecific hybrids by *Hpa*II (left lanes) and *Msp*I (right lanes) digestion. **a**, Hybridization between *Mus spretus* and *M. musculus*. Lane 1, male *M. spretus*; 2, male *M. musculus* × *M. spretus* F₁; 3, male *M. musculus* × *M. spretus* F₁; 4, male *M. musculus* × *M. spretus* F₁; 5, male *M. musculus* × *M. musculus*; 6, female *M. musculus*; 7, female *M. musculus* × *M. spretus* F₁; 8, female *M. musculus* × *M. musculus*; 9, female *M. spretus*. **b**, Hybridization between horse and donkey (mule) and donkey and horse (hinny). Lane 1, female horse; 2, female mule; 3, female mule 2; 4, male donkey; 5, male horse; 6, female hinny; 7, female donkey; 8, female hinny; 9, male horse. In all matings, the female is indicated first. Each lane corresponds to an individual animal. M, marker.

In addition, we assessed cytosine methylation by *in situ* nick-translation on the metaphase chromosomes of mouse F₁ hybrids and parental mice. For this analysis, mice of the *Mus musculus* strain Cremona were used. Because this strain has an aberrant chromosome number (2N = 22 instead of 40), it is possible to discriminate between the chromosomes derived from *Mus musculus* Cremona and *Mus spretus* in F₁ hybrids (Fig. 2). This analysis indicated that no major change had occurred in genome-wide demethylation or in centromere expansion. Finally, we were unable to detect amplification of the L1 retrotransposon by Southern-blot analysis (results not shown).

These results do not appear to agree with those obtained by O'Neill *et al.*¹ in their analysis of interspecific hybrids between several different species of macropodid marsupials. However, their findings are unequivocal and supported by earlier cytogenetic investigations of hybrids between additional macropodid species⁸. Genome-wide undermethylation, retrotransposon

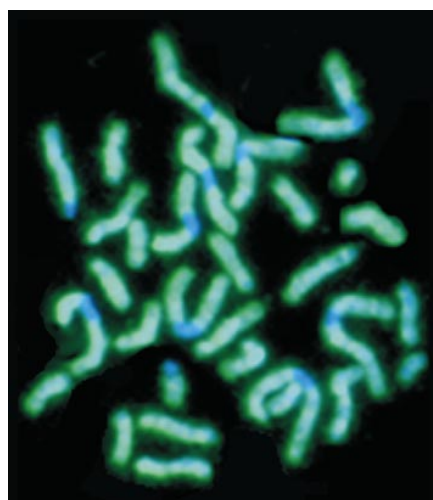


Figure 2 Methylation analysis of *M. musculus* Cremona × *M. spretus* chromosomes. Metaphase spread from bone marrow was digested with *Hpa*II followed by *in situ* nick-translation. Staining patterns were identical with *Msp*I. There was no detectable difference in labelling intensity between *M. spretus* and *M. musculus* euchromatin. The faint labelling of *M. musculus* compared with *M. spretus* centromeric heterochromatin reflects the low frequency of *Msp*I/*Hpa*II sites in *M. musculus* major satellite DNA.

activation and chromosome extension may therefore be specific to interspecific hybridization in marsupials, or perhaps they occur only in macropodid marsupials.

In any case, our results argue against the idea that such profound alterations in genome organization of interspecific hybrids are common events in placental mammals. Marsupials and placental mammals diverged about 130 million years ago⁹, so the functional role of methylation may have changed between the two subclasses. For example, marsupial and placental mammals show pronounced differences in their processes of X-chromosome inactivation⁹, in which the role of methylation is thought to be important¹⁰.

Irmgard Roemer*, **Frank Grützner***, **Heinz Winking†**, **Thomas Haaf***, **Annie Orth‡**, **Lulu Skidmore§**, **Doug Antczak¶**, **Reinald Fundele***

*Max-Planck-Institut für Molekulare Genetik, Ihnestr. 73, 14195 Berlin, Germany
e-mail: fundele@mpimg-berlin-dahlem.mpg.de

†Institut für Biologie, Medizinische Universität Lübeck, 23562 Lübeck, Germany

‡Laboratoire Génome et Populations, Université de Montpellier II, 34095 Montpellier, France

§Camel Reproduction Centre, Dubai, United Arab Emirates

¶James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853, USA

- O'Neill, R. J. W., O'Neill, M. J. & Graves, J. A. M. *Nature* **393**, 68–72 (1998).
- Gray, A. P. in *Mammalian Hybrids* (Commonwealth Agricultural Bureau, Edinburgh, 1971).
- Allen, W. R., Skidmore, J. A., Stewart, F. & Antczak, D. F. *J. Reprod. Fertil.* **9**, 55–60 (1993).

- Rogers, J. F. & Dawson, W. D. *J. Reprod. Fertil.* **21**, 255–262 (1970).
- Zechner, U. *et al.* *Nature Genet.* **12**, 398–403 (1996).
- Orr, H. A. *Annu. Rev. Ecol. Syst.* **28**, 195–218 (1997).
- Templeton, A. R. *Annu. Rev. Ecol. Syst.* **12**, 23–48 (1981).
- Smith, M. J., Hayman, D. L. & Hope, R. M. *Aust. J. Zool.* **27**, 959–972 (1979).
- Graves, J. A. M. *Trends Genet.* **3**, 252–256 (1987).
- Migeon, B. R. *Trends Genet.* **10**, 415–417 (1994).

O'Neill *et al.* reply — The absence of global methylation changes in eutherian interspecific hybrids compared with their parents, observed by Roemer *et al.*, sharply contrasts with our own studies of interspecific hybrids between various species of kangaroo. We observed hybrid-specific undermethylation, retroelement activation and genome remodelling¹, and suggested that these events occurring together may bring about rapid karyotypic change.

We agree with Roemer *et al.* that marsupials and eutherians are likely to have diverged from one another in their reliance on and use of the epigenetic information conveyed by DNA methylation. Although these events may be specific to marsupial or even macropod interspecific hybrids, there is evidence that eutherian genomes may be subject to at least some degree of the same sort of hybrid dysgenic perturbations. Interspecific hybrids of the genus *Peromyscus* (deer mice) do not show whole-genome changes in methylation, as determined by digestion with *Msp*I and *Hpa*II (R.J.W.O'N. *et al.*, unpublished observations), yet they exhibit disruptions in imprinted gene expression associated with allele-specific undermethylation². The mechanism underlying the loss of imprinting in these hybrids remains unknown, but there is a subtle change in methylation in this eutherian hybrid cross². Digestion of genomic DNA with *Msp*I and *Hpa*II may be too blunt an instrument to reveal subtle changes (less than 20%) in methylation.

Our main finding is a link between DNA methylation, retroelement activity and genome rearrangement. The dramatic perturbations of methylation and genome structure that we observed in kangaroo hybrids may be an extreme example of dysgenic changes that occur on a broader scale in many organisms.

Rachel J. Waugh O'Neill*, **Michael J. O'Neill***, **Jennifer A. Marshall Graves†**

*Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269, USA

e-mail: roneill@uconnvm.uconn.edu

†Department of Genetics and Human Variation, La Trobe University, Bundoora, Victoria 3083, Australia

- O'Neill, R. J. W., O'Neill, M. J. & Graves, J. A. M. *Nature* **393**, 68–72 (1998).
- Vrana, P. B. *et al.* *Nature Genet.* **20**, 362–365 (1998).

Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid

Anne Schmidt^{*†}, Michael Wolde[‡], Christoph Thiele^{*}, Werner Fest[‡], Hartmut Kratzin[§], Alexandre V. Podtelejnikov^{||}, Walter Witke^{||}, Wieland B. Huttner^{*} & Hans-Dieter Söling[‡]

^{*} Max-Planck-Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 110, D-01307 Dresden, and Department of Neurobiology, University of Heidelberg, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany

[‡] Department of Clinical Biochemistry, University of Göttingen, and Max-Planck-Institute of Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany

[§] Max-Planck-Institute for Experimental Medicine, Herman-Rein-Strasse 3, D-37075 Göttingen, Germany

^{||} European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69012 Heidelberg, Germany

[†] These authors contributed equally to this work.

Endophilin I is a presynaptic protein of unknown function that binds to dynamin, a GTPase that is implicated in endocytosis and recycling of synaptic vesicles. Here we show that endophilin I is essential for the formation of synaptic-like microvesicles (SLMV) from the plasma membrane. Endophilin I exhibits lysophosphatidic acid acyl transferase (LPAAT) activity, and endophilin-I-mediated SLMV formation requires the transfer of the unsaturated fatty acid arachidonate to lysophosphatidic acid, converting it to phosphatidic acid. A deletion mutant lacking the SH3 domain through which endophilin I interacts with dynamin still exhibits LPAAT activity but no longer mediates SLMV formation. These results indicate that endophilin I may induce negative membrane curvature by converting an inverted-cone-shaped lipid to a cone-shaped lipid in the cytoplasmic leaflet of the bilayer. We propose that, through this action, endophilin I works with dynamin to mediate synaptic vesicle invagination from the plasma membrane and fission.

Membrane traffic mediated by vesicular carriers is a fundamental process in all eukaryotic cells. Any given trafficking step requires two processes of membrane fusion; that leading to the pinching-off of the vesicle from its donor membrane, called fission, and that resulting in the merging of the vesicle with its acceptor membrane. With regard to membrane fission, two principal models can be distinguished. These are not necessarily mutually exclusive but may reflect basic differences between the various types of intracellular donor membranes and vesicles.

One model views the process of membrane fission as the ultimate consequence of membrane budding. Here, the continuation of the change of shape of the donor membrane by a proteinaceous coat machinery that mediates budding is thought to constrict the lipid bilayer at the edge of the polymerizing coat, that is, the neck of the nascent vesicle, sufficiently for membrane fission to occur. Studies on vesicle formation in the early secretory pathway have provided considerable evidence in support of this model, notably the demonstration that the coat proteins (COP I and COP II) are sufficient to generate small lipid vesicles from liposomes, if the liposomes bear an integral membrane constituent (lipid or membrane-anchored protein motif) that is appropriate for coat recruitment^{1–3}.

The other model views membrane budding and membrane fission as being separate, though often linked, processes that are mediated by distinct proteinaceous machineries with the potential, but not the need, to interact. Here, following the coat-mediated formation of a membrane bud, membrane fission results from the action of a separate set of proteins that mediate the constriction of the narrow membrane tubule connecting the nascent vesicle with the donor membrane. Evidence supporting this model has largely been obtained in studies on endocytosis, in particular the recycling of synaptic vesicles from the plasma membrane^{4–8}. Whereas the clathrin coat

mediates bud formation^{7,9}, a ring around the neck of the nascent vesicle, formed upon polymerization of the GTPase dynamin^{10,11}, is thought to be central in membrane constriction and fission^{4–6,8}.

It has been proposed that this membrane constriction and fission is driven by a change in the conformation of dynamin that occurs upon GTP hydrolysis^{4,5,8,11–14}. However, the view that dynamin itself acts as a 'pinchase' has been questioned¹⁵. In fact it appears that dynamin does not function as a force-generating GTPase but rather, like other members of the GTPase superfamily, activates an as yet unknown effector system when in the GTP-state, and it is this effector system that is essential for fission^{16,17}. If so, dynamin-interacting proteins, such as the endophilin I (originally called SH3p4, ref. 18) that is studied here, are obvious candidates for this effector system.

The shape of a membrane is influenced not only by proteins but also by lipids. The shape of membrane lipids is a key determinant of membrane curvature^{19–21}. Inverted-cone-shaped lipids favour positive curvature, which characterizes the outer leaflet of a membrane bud, and cone-shaped lipids favour negative curvature, as found in the inner leaflet of a bud. Examples of inverted-cone-shaped lipids and cone-shaped lipids include lysophospholipids and unsaturated fatty acids, respectively^{20,22}. Indeed, in one of the paradigms of membrane fusion, that of the influenza virus envelope with the plasma membrane mediated by the viral haemagglutinin^{23,24}, the differentially shaped lipids lysophosphatidylcholine (inverted cone) and oleic acid (cone) affect the fusion process in an opposite (negative and positive, respectively) manner²². The shape of these lipids is thought to affect critically the transition between the intermediates in lipid bilayer organization that have been hypothesized to exist during the fusion process^{20,21}.

This raises two questions. First, do differently shaped lipids also have a role in membrane fission? Second, how is lipid shape controlled? Here we report on the role of endophilin I in the control of lipid shape and changes in membrane curvature underlying the formation of synaptic vesicles from the plasma membrane.

[†] Present address: Protein Interaction Laboratory, University of Southern Denmark, Campusvej 55, Odense, DK-5230 Denmark (A.V.P.); Mouse Biology Programme, European Molecular Biology Laboratory, Via Ramarini 32, I-00016 Monterotondo, Italy (W.W.)

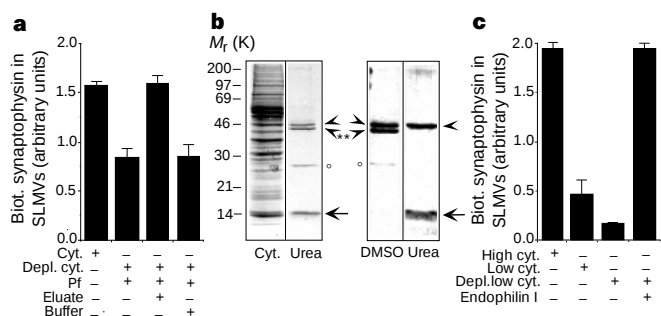


Figure 1 Endophilin I is rate-limiting for SLMV biogenesis. **a**, SLMV formation in perforated PC12 cells using either complete (Cyt.) or poly(L-proline)-depleted (Depl. cyt.) brain cytosol (1.8 mg protein per ml) supplemented as indicated with $3 \mu\text{g ml}^{-1}$ recombinant profilins (Pf, 30:1 ratio of mouse profilin II to human profilin I), dialysed 30% DMSO eluate from the poly(L-proline) column containing $\sim 8 \mu\text{g}$ 40K protein per ml (Eluate), and/or the equivalent amount of dialysed 30% DMSO elution buffer (Buffer). **b**, SDS-PAGE followed by Coomassie-Blue staining of complete brain cytosol (Cyt.), the 8 M urea eluate from the poly(L-proline) column containing $\sim 8 \mu\text{g}$ 40K protein per ml (Urea), the 30% DMSO eluate from the poly(L-proline) column (DMSO), and the 8 M urea eluate from the poly(L-proline) column obtained after elution with 30% DMSO (right, Urea). Arrowheads, actin; arrowheads with asterisk, 40K protein; circles, 28K protein; arrows, profilins. **c**, SLMV formation in perforated PC12 cells using brain cytosol supplemented with $25 \mu\text{g ml}^{-1}$ purified (poly(L-proline)) recombinant His₆-endophilin I as indicated: complete high-protein cytosol (High cyt., $3.75 \text{ mg protein per ml}$) or low-protein cytosol (Low cyt., $0.75 \text{ mg protein per ml}$), poly(L-proline)-depleted low-protein cytosol (Depl. low cyt., $0.75 \text{ mg protein per ml}$). In **a** and **c**, data are the mean of two independent perforated-cell reactions; error bars indicate the variation of the individual values from the mean.

Endophilin I is required for SLMV formation

To identify rate-limiting factors for SLMV formation, we used a previously established perforated-cell system, derived from the rat neuroendocrine cell line PC12, which reconstitutes this process²⁵. This perforated-cell system is based on cell-surface biotinylation of the synaptic vesicle membrane protein synaptophysin, and its accumulation in a specialized domain of the plasma membrane at 18°C (ref. 26), followed by its incorporation into SLMVs upon cell perforation, addition of brain cytosol and ATP, and a return of the temperature to 37°C . In investigating whether the dynamin-interacting²⁷, actin-binding protein profilin was involved in SLMV formation, we noted that cytosol depleted of profilin by passage over poly(L-proline) Sepharose²⁸ was much less efficient than control cytosol but that addition of recombinant profilin to the depleted cytosol did not fully restore SLMV formation (Fig. 1a). This indicated that passage of cytosol over poly(L-proline) Sepharose led to the depletion of a protein (or proteins) other than profilin that is required for SLMV formation.

Elution of the proteins bound to poly(L-proline) Sepharose with 8 M urea revealed four major bands with relative molecular masses (M_r) of 45K, 40K, 28K and 15K (Fig. 1b). Immunoblotting (data not shown) revealed the identity of the 45K and the 15K bands as actin and profilin, respectively. Selective elution of the 40K protein and the 28K protein, along with some actin but without profilin, was achieved using 30% dimethyl sulphoxide (DMSO) (Fig. 1b). Addition of the DMSO eluate to depleted cytosol supplemented with recombinant profilin fully restored SLMV formation (Fig. 1a).

High-accuracy matrix-assisted laser desorption/ionization (MALDI) peptide-mass mapping revealed that the 40K protein was endophilin I (originally called SH3p4, refs 8, 18, 29), with 51%, 48% and 44% of the complete human (accession no. Q99962), complete mouse (accession no. Q62420) and partial rat (accession no. AF009603) sequence, respectively, being covered. Similarly, the 28K protein was unambiguously identified as the SH3- and SH2-domain-containing protein Grb2 (data not shown).

Endophilin I interacts with dynamin³⁰, synaptotagmin^{30,31} and amphiphysin²⁹, which have been implicated in synaptic vesicle

Table 1 Purification of LPAAT from bovine brain

Purification step	Protein (mg)	Total LPAAT activity (mU)	Specific LPAAT activity (mU mg ⁻¹ protein)
Extract	16,520	1,962	0.12
Q-Sepharose (peak II)	5,284	1,532	0.29
S-Sepharose	480	566	1.18
Mono Q HR	129	122	0.95
Superdex 75	8.8	59	6.65
Mono Q HR	5.8	37	6.38
ESPACE	3.3	55	16.51

recycling^{6,8,32}. The identification of the 40K protein as endophilin I indicated that this protein might be the relevant component in the DMSO eluate with regard to SLMV formation. We therefore investigated the effect of recombinant endophilin I on SLMV formation in the perforated-cell system using low-protein cytosol (cytosol that is suboptimal for SLMV formation²⁵). SLMV formation in the presence of low-protein cytosol that had been completely depleted of endogenous endophilin I by passage over poly(L-proline) Sepharose (as revealed by immunoblotting; data not shown) was almost as low (Fig. 1c) as the background value obtained in the absence of added cytosol. Addition to depleted cytosol of a concentration of recombinant endophilin I corresponding to that in high-protein cytosol increased SLMV formation in the perforated-cell system to that observed with high-protein cytosol²⁵ (Fig. 1c). This showed not only that endophilin I is the critical cytosolic component depleted by passage over poly(L-proline) Sepharose, but also that this protein is rate-limiting for SLMV formation. Confirming the latter conclusion, addition of increasing amounts of recombinant endophilin I (up to $16 \mu\text{g ml}^{-1}$) to low-protein cytosol resulted in a progressive stimulation of SLMV formation, which approached the level obtained with high-protein cytosol (data not shown). SLMV formation was also stimulated by recombinant endophilin I in a modified cell-free system, in a similar manner to the perforated-cell system (data not shown). In this system, PC12 cells were biotinylated at 18°C and homogenized (rather than only perforated), and the homogenate was subjected to cell-free reaction followed by isolation of SLMVs without further homogenization (analogous to the cell-free system of ref. 33).

Endophilin I has LPAAT activity

An independent line of investigation originated from the observations^{34–36} that stimulation of exocytosis in parotid acinar cells is associated with an increase in the activity of lysophosphatidic acid acyl transferase (LPAAT), which converts lysophosphatidic acid (LPA) to phosphatidic acid. The increase in LPAAT activity upon stimulation of secretion is due to phosphorylation by cyclic-AMP-dependent protein kinase or Ca^{2+} /calmodulin-dependent protein kinase II (refs 35, 36). To identify this phosphorylation-sensitive LPAAT, we purified it (Table 1). Bovine brain tissue was used as the source because LPAAT activity increases upon addition of either protein kinase to a brain-derived total post-nuclear membrane fraction³⁶. Q-Sepharose ion-exchange chromatography gave two peaks of LPAAT activity, one (peak I) of which also contained lysophosphatidylcholine (LPC) acyl transferase activity and was not significantly stimulated by phosphorylation, and the other (peak II) of which did not exhibit significant LPC acyl transferase activity but was stimulated by phosphorylation (data not shown). LPAAT in peak II was purified to apparent homogeneity (Table 1). The final purification step consisted of preparative, continuous elution SDS-polyacrylamide gel column electrophoresis (ESPACE). The fractions obtained were examined for protein content by analytical SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2a) and for LPAAT activity after protein renaturation (Fig. 2b). This revealed a single $\sim 40\text{K}$ protein band (Fig. 2a), which coincided with LPAAT activity (Fig. 2b), in fractions 18–30. Two-dimensional (2D) PAGE showed that the 40K protein band consisted of a single protein with

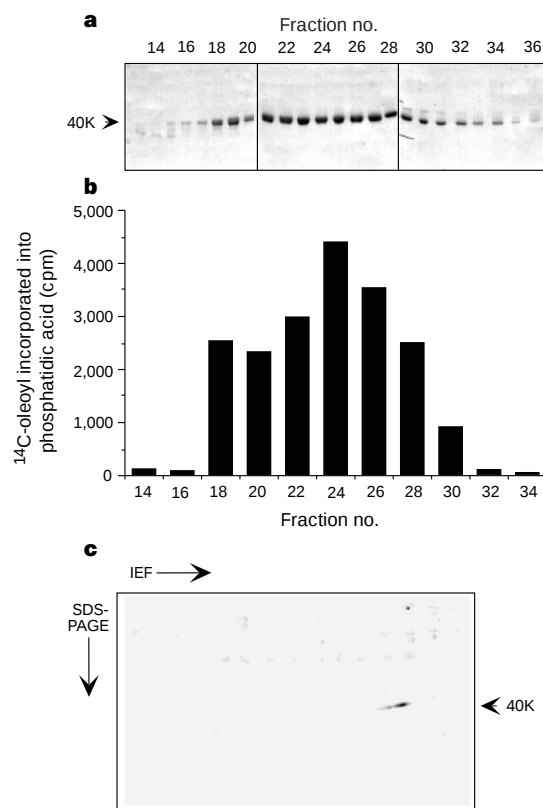


Figure 2 Identification of purified bovine brain LPAAT as endophilin I. **a**, **b**, ESPACE, the final step in the purification. Aliquots of some of the ~150 fractions obtained after ESPACE were subjected to analytical SDS-PAGE on three minigels followed by Coomassie-Blue staining (**a**, entire separating gels are shown), and the remainder of the indicated fractions was renatured and aliquots of equal volume (containing 3.3 µg protein in the case of fraction 24) analysed for LPAAT activity (**b**). **c**, 2D PAGE followed by Coomassie-Blue staining of the pooled fractions containing the peak of LPAAT activity (5 µg protein).

an acidic isoelectric point (Fig. 2c). The sequence of five tryptic peptides obtained from the purified 40K protein (AVMEIMTKT IEYL, IRGQEKGPYPQAEA, GPGYPQAEALLAEAML, QGKIPD EELRQALE, EIAESSMFNLLEMD) was identical to that of predicted tryptic fragments of endophilin I. This, together with the finding that the reported molecular mass (40K, refs 30, 31) and predicted isoelectric point (pI 5.33) of endophilin I are, within the range of experimental accuracy, identical to those of the 40K band that comigrates with LPAAT activity upon purification, indicates that LPAAT activity is intrinsic to endophilin I.

When oleoyl-CoA was used as a co-substrate, the purified bovine brain enzyme exhibited a high specificity for LPA as substrate as compared to other lysophospholipids (Table 2). Moreover, like the enzyme studies in tissue^{34–36} and crude subcellular fractions^{35,36}, the activity of the purified enzyme was stimulated by phosphorylation with cAMP-dependent protein kinase (Table 2) or Ca²⁺/calmodulin-dependent protein kinase II (data not shown). Activation of the purified enzyme was accompanied by [³²P]phosphate incorporation into the 40K band that approached a value of 1 mol of phosphate per

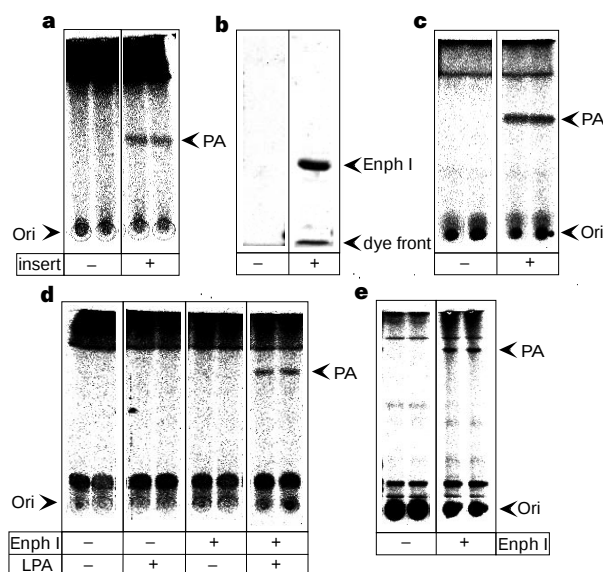


Figure 3 Endophilin I exhibits LPAAT activity. **a**, **c**, Duplicate LPAAT assays using 5 µM [¹⁴C]arachidonoyl-CoA, 10 µM LPA and protein from a lysate (freezing/thawing) of bacteria transformed without (insert –) or with (insert +) the insert coding for His₆-endophilin I; **a**, 280,000g supernatant (~0.5 mg ml⁻¹ protein); **c**, 15 µg ml⁻¹ purified (poly(L-proline)) recombinant His₆-endophilin I. **b**, Coomassie-Blue stained SDS-polyacrylamide gel of the purified recombinant His₆-endophilin I (~10 µg protein) used in **c**. **d**, Duplicate LPAAT assays using 5 µM [¹⁴C]arachidonoyl-CoA in the absence (–) and presence (+) of 10 µM LPA and 10 µg ml⁻¹ recombinant (metal-chelate) His₆-endophilin I as indicated. **e**, Duplicate LPAAT assays using 50 µM [¹⁴C]arachidonoyl-CoA and 100 µM LPA in the absence (–) and presence (+) of the 30% DMSO eluate (~5 µg endophilin I per ml) obtained from the poly(L-proline) column loaded with brain cytosol. **a–e**, Enph I, endophilin I; PA, phosphatidic acid; Ori, origin.

mol of protein (data not shown).

To confirm that endophilin I has LPAAT activity, recombinant endophilin I was expressed in bacteria as either His₆-tagged or glutathione S-transferase (GST)-fusion protein and analysed for LPAAT activity. To exclude a contribution of endogenous bacterial LPAAT, which is a transmembrane protein³⁷, the bacteria were lysed by freezing-thawing in the absence of detergent, followed by ultracentrifugation. The high-speed supernatant obtained from bacteria transformed with vector containing the insert encoding His₆-tagged endophilin I exhibited LPAAT activity in the presence of LPA and arachidonoyl-CoA as exogenous substrates, whereas that obtained after transformation with vector lacking the insert did not (Fig. 3a). The same results were obtained when oleoyl-CoA was used instead of arachidonoyl-CoA (data not shown).

Recombinant His₆-tagged endophilin I was purified from the bacterial high-speed supernatant by affinity chromatography on poly(L-proline) Sepharose (Fig. 3b). Purified recombinant endophilin I exhibited LPAAT-activity (Fig. 3c). In contrast, a poly(L-proline) eluate obtained from the high-speed supernatant of bacteria transformed with vector lacking insert, which did not contain endophilin I (Fig. 3b), did not show detectable LPAAT activity (Fig. 3c). Similar results were obtained with recombinant His₆-tagged endophilin I purified by metal chelate affinity chromato-

Table 2 Lysophospholipid specificity of purified and recombinant endophilin I and its activation by phosphorylation

Protein	LPA		LPC	LPS	LPI	LPE
	Control	PKA				
Purified endophilin I	100	306	5.8	7.2	1.0	5.5
His ₆ -endophilin I	100	204	4.2	3.9	0.9	1.5
GST-endophilin I	100	205	3.3	3.3	2.6	3.2

Specific LPAAT activity is expressed as percentage of control, using LPA as substrate. Specific LPAAT activities for the control condition were 4.95, 10.80 and 5.46 for endophilin I purified from bovine brain, His₆-endophilin I and GST-endophilin I, respectively. PKA, cyclic AMP-dependent protein kinase.

graphy, and with a GST–endophilin-I fusion protein purified from bacterial high-speed supernatant by affinity chromatography on either GSH-agarose or poly(L-proline) Sepharose (data not shown).

The LPAAT activity of recombinant, His₆-tagged or GST-fused endophilin I, purified by affinity chromatography on metal chelator or GSH-matrices, respectively, was dependent on the presence of exogenous LPA (Fig. 3d and data not shown) and was stimulated by phosphorylation with cAMP-dependent protein kinase (Table 2). Purified recombinant endophilin I showed essentially the same specificity for LPA as substrate, as compared to other lysophospholipids, as did the enzyme purified from bovine brain (Table 2).

Given the original observation that the poly(L-proline) DMSO eluate obtained from brain cytosol, which was highly enriched in endophilin I (Fig. 1b), promoted SLMV formation in the perforated-cell system (Fig. 1a), it was important to verify that this eluate exhibited LPAAT activity. This was indeed the case (Fig. 3e).

Endophilin I binds fatty acyl-CoA and LPA

Endophilin I was depleted from brain cytosol upon passage over a palmitoyl-CoA agarose column, in contrast to the bulk of the cytosolic proteins and Grb2 (Fig. 4a), another SH3-domain-containing protein with an isoelectric point similar to that of endophilin I.

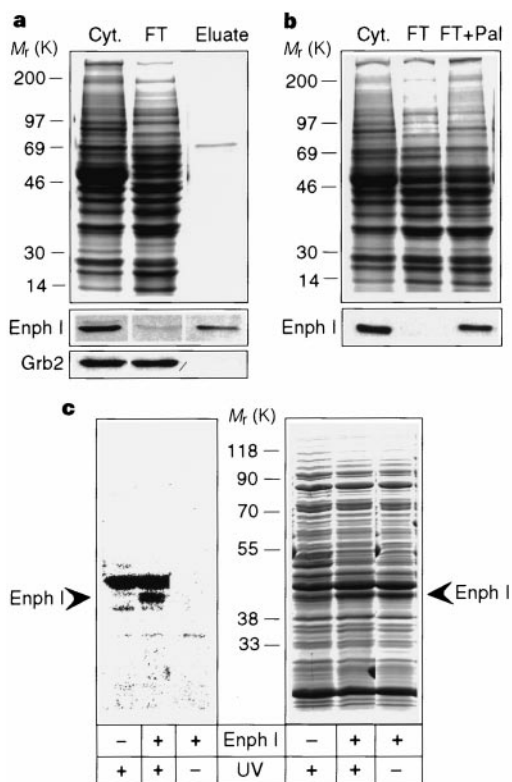


Figure 4 Endophilin I binds to fatty acyl-CoA and LPA. **a, b**, Binding of endophilin I from brain cytosol to palmitoyl-CoA agarose. Top panels: Coomassie-Blue-stained SDS-polyacrylamide gel of the cytosol loaded onto palmitoyl-CoA agarose (Cyt.), the corresponding flow-through obtained in the absence (**a, b**, FT) or presence (**b**, FT + Pal) of 40 mM free palmitoyl-CoA added to the cytosol before loading, and the eluate obtained upon addition of 4 mM free palmitoyl-CoA (**a**, Eluate). Bottom panels: Endophilin I (Enph I) and Grb2 immunoblots of the material shown in the top panels. In **a**, the eluate corresponds to 1/4 of the loaded cytosol and flow-through and, therefore, the exposure time of the endophilin I immunoblot for the eluate is four times that of the cytosol and flow-through. **c**, Photoaffinity labelling of recombinant endophilin I using [³²P]1-(10-azido-stearoyl)-LPA. The 280,000g supernatant obtained from a lysate (freezing/thawing) of bacteria transformed without (Enph I-) or with (Enph I+) the insert coding for His₆-endophilin I was incubated in the presence of [³²P]1-(10-azido-stearoyl)-LPA without (UV-) or with (UV+) UV irradiation, followed by SDS-PAGE, Coomassie-Blue staining (right panel) and phosphorimaging (left panel).

philin I. Binding of endophilin I to palmitoyl-CoA agarose was prevented when cytosol was loaded onto the column in the presence of free palmitoyl-CoA (Fig. 4b). Likewise, endophilin I bound to palmitoyl-CoA agarose was eluted from the column by free palmitoyl-CoA (Fig. 4a). These results indicate that endophilin I binds fatty acyl-CoA.

To investigate the binding of endophilin I to LPA, we prepared a radioactive photoactivatable analogue of LPA, [³²P]1-(10-azido-stearoyl)-LPA. When a high-speed supernatant obtained from bacteria transformed with vector containing the insert encoding His₆-tagged endophilin I was subjected to UV-irradiation in the presence of the photoactivatable LPA, a ³²P-labelled 40K band was observed (Fig. 4c, left) that coincided with recombinant endophilin I detected by protein staining (Fig. 4c, right). Labelling of this band was dependent on UV-irradiation (Fig. 4c) and was blocked by excess unlabelled LPA (data not shown). The 40K band was not detected when a high-speed supernatant from bacteria transformed with vector lacking insert was used, in contrast to an endogenous bacterial 45K protein that was equally labelled in both supernatants (Fig. 4c). Similar photoaffinity labelling was observed for the GST–endophilin-I fusion protein (data not shown). We conclude that endophilin I binds LPA.

Effects of added LPA and fatty acyl-CoA

Addition of 1 μM LPA to the perforated-cell system did not significantly affect the basal SLMV formation observed with low-protein cytosol, but completely blocked the ability of exogenously added recombinant endophilin I to mediate SLMV formation (Fig. 5a). This is consistent with the interpretation that exogenously added LPA competitively prevents endophilin I from acting on LPA in the donor membrane.

To investigate the requirement for fatty acyl-CoA in SLMV formation mediated by endophilin I, we used the unsaturated

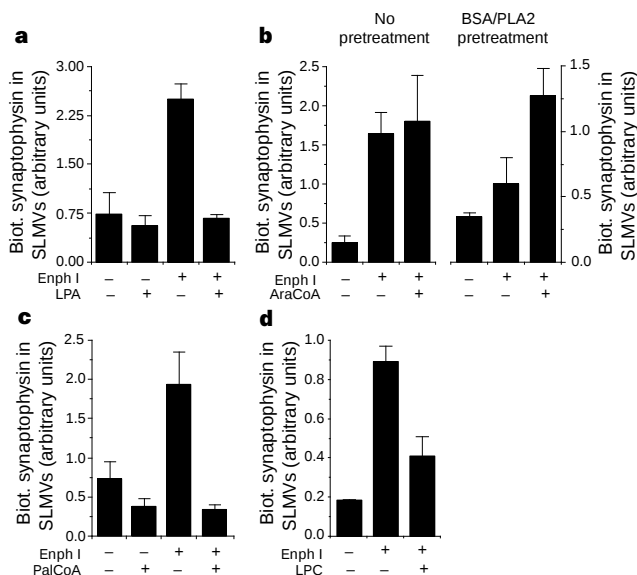


Figure 5 Effects of lysophospholipids and fatty acyl-CoA on endophilin-I-mediated SLMV formation. SLMV formation using perforated PC12 cells without (**a, b** left, **c, d**) or with (**b** right) BSA/PLA₂ pretreatment, supplemented with complete (and, for **b** and **c**, gel-filtered) brain cytosol at 0.75 mg protein per ml (**a, b** left, **c, d**) or 2.5 mg protein per ml (**b** right), in the absence (-) or presence (+) of the indicated additions: **a**, 1 μM LPA, 10 μg ml⁻¹ purified (metal-chelate) recombinant His₆-endophilin I; **b**, 10 μM arachidonoyl-CoA (AraCoA), 10 μg ml⁻¹ purified (metal-chelate and poly(L-proline)) recombinant His₆-endophilin I; **c**, 10 μM palmitoyl-CoA (PalCoA), 10 μg ml⁻¹ purified (metal-chelate) recombinant His₆-endophilin I; **d**, 2 μM LPC, 10 μg ml⁻¹ purified (poly(L-proline)) recombinant His₆-endophilin I. **a–d**, Data are the mean of three (**a, b** left, **c, d**) or two (**b** right) independent perforated-cell reactions; bars indicate s.d. or the variation of the individual values from the mean. Enph I, endophilin I.

fatty acid arachidonoyl-CoA rather than the saturated fatty acid palmitoyl-CoA because, upon stimulation of secretion, the increase in incorporation of arachidonate into phosphatidic acid is greater than that of palmitate³⁴. In the standard perforated-cell system using gel-filtered low-protein cytosol, addition of 10 μ M exogenous arachidonoyl-CoA in the presence of recombinant endophilin I did not increase SLMV formation above the level obtained by addition of endophilin I alone (Fig. 5b, left). Likewise, arachidonoyl-CoA did not increase the basal SLMV formation obtained without addition of endophilin I (data not shown). This indicated that, under these conditions, endogenous arachidonoyl-CoA was not rate-limiting. Presumably, in the presence of gel-filtered cytosol, the source of fatty acyl-CoA was fatty acid derived from membrane phospholipids through endogenous phospholipase A₂ (PLA₂) followed by activation by cytosolic fatty acyl-CoA synthase.

To remove free fatty acids and fatty acyl-CoA from the membranes, the perforated cells were pretreated with fatty acid-free bovine serum albumin (BSA). Including PLA₂ under non-lytic conditions (see Methods) during the BSA pretreatment reduced basal SLMV formation in the subsequent perforated-cell reaction to about half of that observed after BSA pretreatment in the absence of added PLA₂; addition of 10 μ M arachidonoyl-CoA during the perforated-cell reaction following BSA/PLA₂ pretreatment restored basal SLMV formation to the original level (data not shown). We therefore used this pretreatment to show that arachidonoyl-CoA is required for endophilin-I-mediated SLMV formation. Compared to the marked stimulation of SLMV formation by endophilin I seen without BSA/PLA₂ pretreatment (Fig. 5b left), BSA/PLA₂ pretreatment greatly reduced endophilin-I-mediated SLMV formation in the absence of exogenously added arachidonoyl-CoA (Fig. 5b right); addition of 10 μ M arachidonoyl-CoA doubled the endophilin-I-mediated SLMV formation (Fig. 5b right). We conclude that endophilin-I-mediated SLMV formation requires fatty acyl-CoA.

We next investigated whether palmitoyl-CoA, which, like arachi-

donoyl-CoA, can be used as substrate by endophilin I to generate phosphatidic acid (data not shown), also supported endophilin-I-mediated SLMV formation. In the standard perforated-cell system using low-protein cytosol, addition of 2.5 μ M (data not shown) or 10 μ M (Fig. 5c) of palmitoyl-CoA completely blocked endophilin-I-mediated SLMV formation, in contrast to arachidonoyl-CoA (Fig. 5b). Addition of 2 μ M LPC, which was a much poorer substrate for endophilin I than LPA (Table 2), also inhibited endophilin-I-mediated SLMV formation (Fig. 5d).

A PLA₂ inhibitor potentiates SLMV formation

If SLMV formation mediated by endophilin I is due to the conversion of LPA to phosphatidic acid, one might expect that a PLA₂ inhibitor acts synergistically with endophilin I in SLMV formation because the reverse reaction, the hydrolysis of phosphatidic acid to LPA and free fatty acid, which presumably occurs during the perforated-cell reaction owing to the activity of cytosolic PLA₂, would be prevented. Indeed, the specific inhibitor of cytosolic calcium-dependent and -independent PLA₂, methyl arachidonyl fluorophosphonate (MAFP³⁸), strongly potentiated endophilin-I-mediated SLMV formation (Fig. 6a). This observation indicates that endogenous cytosolic PLA₂ may be able to antagonize the action of endophilin I in SLMV formation.

In most of the experiments described above, the effects of recombinant endophilin I on SLMV formation were investigated in the presence of low-protein cytosol, which by itself supported a basal level of SLMV formation. Given the results of Fig. 1c, this basal SLMV formation can largely be attributed to the endophilin I endogenously present in low-protein cytosol ($\sim 6 \mu\text{g ml}^{-1}$). Like the SLMV formation mediated by addition of exogenous endophilin I (Fig. 6a), the basal SLMV formation should therefore be sensitive to agents affecting hydrolysis of phosphatidic acid. Indeed, addition of MAFP moderately stimulated basal SLMV formation (Fig. 6b). Conversely, addition of exogenous PLA₂ derived from snake venom markedly reduced basal SLMV formation (Fig. 6c), the residual level being only slightly above that observed without addition of any cytosol (data not shown).

The role of the SH3 domain of endophilin I

Dynamin, which is an essential component of the machinery that mediates SLMV formation in our perforated-cell system²⁵, is recruited to, and forms rings at, the necks of synaptic vesicles budding from their donor membrane⁴. Endophilin I binds, by its SH3 domain, to the proline-rich region of dynamin³⁰. We therefore investigated whether SLMV formation mediated by endophilin I involved protein-protein interaction through its SH3 domain. A $\sim 34\text{K}$ deletion mutant of endophilin I lacking the SH3 domain (referred to as del-endophilin I) still exhibited LPAAT activity (Fig. 7a) but no longer mediated SLMV formation, at least not at the concentration tested ($\sim 10 \mu\text{g ml}^{-1}$; Fig. 7b). This shows that protein-protein interaction of endophilin I through its SH3 domain is essential for its ability to mediate SLMV formation. Although endophilin I binds not only to dynamin³⁰ but also synaptojanin^{30,31}, we interpret the need for its SH3 domain in terms of an interaction with dynamin rather than synaptojanin because, in perforated PC12 cells supplemented with cytosol depleted of both dynamin and synaptojanin, addition of dynamin alone is sufficient to restore SLMV formation²⁵. Specifically, we propose that the interaction with dynamin results in the recruitment of endophilin I, and hence its LPAAT activity, to the membrane of budding synaptic vesicles.

Discussion

We have provided several lines of evidence that endophilin I has LPAAT activity. First, endophilin I isolated from brain using ESPACE as the final purification step appears as a single 40K protein on analytical SDS- and 2D PAGE and exhibits LPAAT activity after

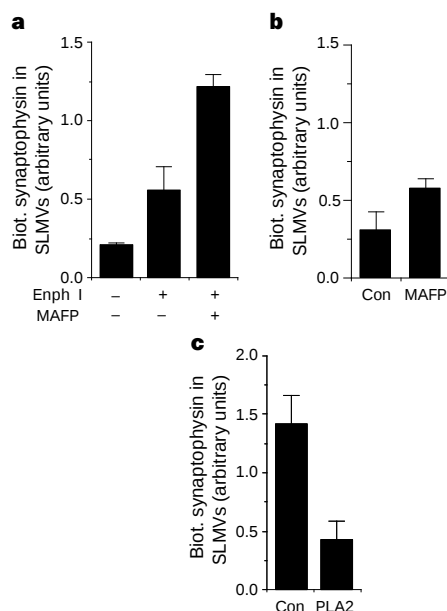


Figure 6 Effects of PLA₂ and the PLA₂ inhibitor MAFP on endophilin-I-mediated SLMV formation. SLMV formation in perforated PC12 cells using complete brain cytosol (0.75 mg protein per ml) in the absence (–, Con) and presence (+) of the indicated additions: **a**, 10 $\mu\text{g ml}^{-1}$ purified (metal-chelate) recombinant His₆-endophilin I (Enph I), 10 μM MAFP; **b**, 10 μM MAFP; **c**, 10 $\mu\text{g ml}^{-1}$ PLA₂ from *C. adamanteus*. Note that to quantify the reduction by PLA₂ below the control, the immunoblot was exposed longer than in the case of a stimulation above the control (such as **a**, **b**), resulting in higher than usual arbitrary units for the control value. **a–c**, Data are the mean of three independent perforated-cell reactions; bars indicate s.d.

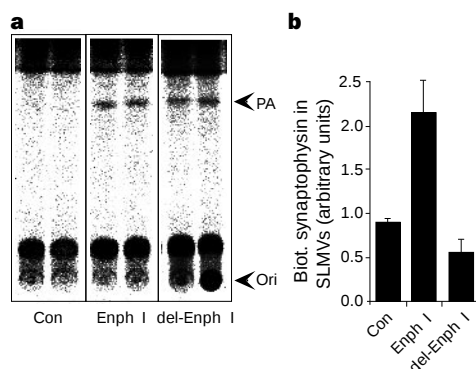


Figure 7 Endophilin I lacking its SH3 domain (del-endophilin I) still possesses LPAAT activity but does not promote SLMV biogenesis. **a**, Duplicate LPAAT assays using 5 μ M [14 C]arachidonoyl-CoA and 10 μ M LPA in the absence (Con) and presence of 10 μ g ml $^{-1}$ purified (metal-chelate) recombinant His $_6$ -endophilin I (Enph I) and del-His $_6$ -endophilin I (del-Enph I). PA, phosphatidic acid; Ori, origin. **b**, SLMV formation in perforated PC12 cells using complete brain cytosol (0.75 mg protein per ml) in the absence (Con) and presence of 10 μ g ml $^{-1}$ purified (metal-chelate) recombinant His $_6$ -endophilin I and del-His $_6$ -endophilin I. Data are the mean of three independent perforated-cell reactions; bars indicate s.d.

renaturation. The protein clearly eluted with a higher apparent molecular mass than the transmembrane LPAATs described so far^{39,40}; all of the latter migrate with apparent relative molecular masses of ~29–31K, and no such band was detected in the fractions containing the purified endophilin I and exhibiting LPAAT activity. Second, recombinant endophilin I exhibited LPAAT activity after various types of purification (GSH-column for GST-endophilin-I, metal-chelate column for His $_6$ -endophilin-I, poly(L-proline) column for either fusion protein). Moreover, recombinant endophilin I was solely responsible for the LPAAT activity observed in the soluble fraction obtained from transformed bacteria lysed by freezing/thawing. Third, endophilin I binds to palmitoyl-CoA and was photoaffinity-labelled by a photoactivatable LPA analogue, showing that it physically interacts with its two substrates.

A phosphorylation-induced increase in LPAAT activity, apparently reflecting the stimulation of LPAAT activity in response to secretagogue originally observed in parotid acinar cells³⁴, has been observed in a postnuclear membrane fraction³⁶. This led us to anticipate that this LPAAT activity would reside in a membrane-associated protein. We therefore used a crude membrane fraction as a source for LPAAT purification. How, then, can it be explained that this purification yielded endophilin I, which upon hypo-osmotic lysis of synaptosomes is largely recovered in the soluble fraction³¹, consistent with it being a cytosolic protein? Presumably, the crude membrane fraction used as starting material for the solubilization of LPAAT, which was obtained from a relatively low-speed iso-osmotic supernatant, still contained synaptosomes (sealed nerve terminals) which, in line with the presynaptic localization of endophilin I (ref. 30), are enriched in this protein.

The observations that low-protein cytosol depleted of endophilin I barely supported SLMV formation in the perforated-cell system and that addition of recombinant endophilin I to this cytosol restored SLMV formation to the level obtained with high-protein cytosol indicate an essential role for endophilin I in this process. The function of endophilin I in SLMV formation appears to be mediated by its LPAAT activity. First, under conditions designed to lower the endogenous fatty acyl-CoA content of the perforated cells, endophilin-I-mediated SLMV formation was largely dependent on exogenous arachidonoyl-CoA. Second, exogenous LPA blocked endophilin-I-mediated SLMV formation, presumably by preventing the action of endophilin I on the endogenous LPA in the SLMV donor membrane. Third, endophilin-I-mediated SLMV formation was strongly promoted by the PLA $_2$ inhibitor MAFP, which would

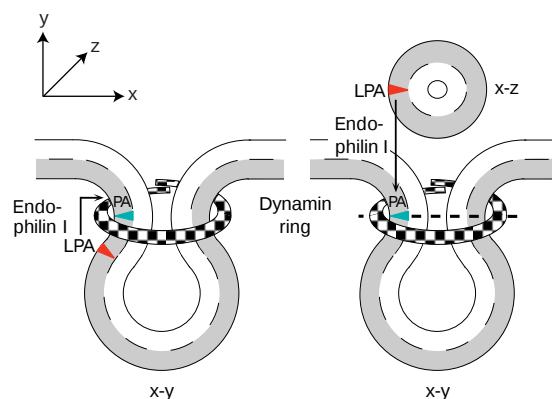


Figure 8 Role of endophilin-I-mediated conversion of LPA to phosphatidic acid (PA) in one of the steps of synaptic vesicle formation (fission). Left: nascent vesicle with neck surrounded by a dynamin ring (drawn in line with its snake¹⁷ or Ferrari⁴⁹ function in endocytosis). The cytoplasmic leaflet of the lipid bilayer is shaded. Owing to its interaction with dynamin I (ref. 30), endophilin I is recruited to the neck of the nascent vesicle. The LPAAT activity of endophilin I (arrow) converts LPA by addition of arachidonate to PA, thereby changing an inverted-cone-shaped lipid inducing positive membrane curvature (red wedge) to a cone-shaped lipid inducing negative curvature (blue wedge) in the cytoplasmic leaflet. This may promote the transition from the bud (positive curvature) to the neck (negative curvature in the spatial x–y plane shown) that precedes fission. Right: in addition to the same view of the nascent vesicle as in the left panel, a cross-section at the neck (dashed line) is shown at the top. This illustrates the positive membrane curvature that exists in the cytoplasmic leaflet (shaded) at the neck in the spatial plane of the cross-section (x–z), in contrast to the negative curvature found upon 90° rotation of this spatial plane (x–y). The endophilin-I-catalysed conversion (arrow) of the positive-curvature-inducing lipid LPA (red wedge) to the negative-curvature-inducing lipid PA (blue wedge) may promote the constriction of the neck towards fission. Note that in addition to fission, other steps in vesicle formation (not illustrated) such as coated pit invagination are also associated with a positive-to-negative change in membrane curvature and that endophilin-I-mediated LPA-to-PA conversion may also operate in these steps.

stabilize the effect of the LPAAT reaction catalysed by endophilin I, that is, an increase in the phosphatidic acid/LPA ratio.

There are several potential mechanisms by which the LPAAT activity of endophilin I could mediate SLMV formation. One is that the phosphatidic acid generated by endophilin I in the donor membrane either stimulates, by activating phosphatidylinositol-4-phosphate (PtdIns(4)P) 5-kinase⁴¹, the formation of PtdIns(4,5)P $_2$, or increases phosphoinositide levels by serving as a precursor in their synthesis. A local increase in the PtdIns(4,5)P $_2$ level in the donor membrane may promote the recruitment of dynamin via its pleckstrin-homology domain, and thereby stimulate SLMV formation^{8,41,42}. Phosphatidic acid may also directly promote dynamin-mediated changes in membrane shape, given that an increase in the amount of phosphatidic acid in liposomes favours the formation of dynamin-coated tubules¹³. However, we have observed that addition of phospholipase D (PLD), which increases phosphatidic acid by hydrolysing phosphatidylcholine, does not increase SLMV formation in the perforated-cell system although, in parallel experiments using another cell-free system, it did increase secretory vesicle formation from the trans-Golgi network (data not shown), confirming previous observations⁴³. This may indicate that endophilin I mediates SLMV formation not simply because it increases phosphatidic acid, but also because it concomitantly consumes LPA.

This leads us to consider changes in membrane curvature as a central feature of the mechanism by which endophilin I mediates the formation of SLMVs and, by extrapolation, the recycling of synaptic vesicles. Synaptic vesicles are the smallest physiologically occurring vesicles of biological membranes known. The neck of a budding synaptic vesicle, especially when this budding occurs from the planar plasma membrane, is therefore characterized by greater membrane curvature than that of other membrane vesicles. Speci-

fically, the positive membrane curvature that exists in the cytoplasmic leaflet of a coated pit and bud changes to negative membrane curvature in one of the two spatial dimensions: at the edge of the coated pit as it invaginates from the planar plasma membrane to form a bud, and at the constricting neck of a bud proceeding to fission from either the planar plasma membrane or a tubular plasma membrane invagination. Inverted-cone-shaped membrane lipids and cone-shaped membrane lipids induce positive and negative membrane curvature, respectively^{19,20}. LPA is thought to be an inverted-cone-shaped lipid, and phosphatidic acid (especially with arachidonate in position 2 of the glycerol backbone) a cone-shaped lipid²⁰. Moreover, inverted-cone-shaped lipids and cone-shaped lipids have been proposed to affect membrane fusion in a differential manner^{20,22}. It is therefore conceivable that endophilin I mediates synaptic vesicle formation because the conversion of LPA to an appropriate phosphatidic acid, catalysed by the LPAAT activity of endophilin I, induces the positive-to-negative membrane curvature change in the cytoplasmic leaflet that is required for coated pit invagination and, as shown in Fig. 8, the constriction of the neck of a budding synaptic vesicle during fission.

Such a mechanism would explain why the unsaturated fatty acid arachidonoyl-CoA supported endophilin-I-mediated SLMV formation, whereas the saturated fatty acid palmitoyl-CoA blocked this process. In line with its ability to use palmitoyl-CoA as substrate (data not shown), endophilin I may have generated a phosphatidic acid with palmitate in position 2. Such a phosphatidic acid may be non-productive for SLMV formation because its generation from LPA would result in a much less pronounced shape change of the lipid from inverted cone to cone than that upon addition of arachidonate.

Alternatively, palmitoyl-CoA, which, in contrast to arachidonoyl-CoA, is an inverted-cone-shaped lipid, may have blocked endophilin-I-mediated SLMV formation not as a substrate but by acting directly on the lipid bilayer, which would imply a profound sensitivity of SLMV formation to lipid shape. Direct evidence for an inhibition of endophilin-I-mediated SLMV formation by inverted-cone-shaped lipids was obtained using LPC (C10:0 in position 1). LPC is not a substrate for endophilin I and hence would presumably not interfere with endophilin-I-catalysed conversion of LPA to phosphatidic acid.

Previous models of dynamin function have suggested that this protein itself may operate as a 'pinchase' at the neck of synaptic and other endocytic vesicles^{5,8}. However, evidence has been reported¹⁶ that dynamin does not function as a force-generating GTPase but rather, like other members of the GTPase superfamily, activates a downstream effector. Our observations raise the possibility that endophilin I constitutes this effector, which would be recruited to the membrane, and perhaps activated, by dynamin to mediate fission. Consistent with this concept, the deletion mutant of endophilin I lacking the SH3 domain, which still exhibited LPAAT activity but presumably no longer bound to dynamin, did not mediate SLMV formation. Activation of endophilin I upon its dynamin-mediated recruitment to the membrane would also explain why the rate of acyl transfer of soluble endophilin I was relatively low ($\leq 17 \text{ mU mg}^{-1} \text{ protein}$).

In mammals, endophilin I belongs to a protein family whose other members are endophilin II and endophilin III (originally called SH3p8 and SH3p13, respectively), which also interact with dynamin³⁰. Like endophilin I, whose tissue distribution is indistinguishable from that of dynamin I, endophilins II and III co-distribute with dynamins II and III, respectively³⁰, consistent with the existence of endophilin I–dynamin I, endophilin II–dynamin II and endophilin III–dynamin III complexes. This raises the possibility that the mechanism of positive-to-negative membrane curvature change in vesicle formation that we propose (the conversion of an inverted-cone-shaped lipid to a cone-shaped lipid by addition of a fatty acyl chain to a lyso-glycerol-lipid) operates not only in SLMV/synaptic vesicle formation mediated by endophilin I–dyna-

min I but, perhaps, also in the formation of other vesicles by endophilin II–dynamin II and endophilin III–dynamin III. Furthermore, this mechanism (operating not necessarily only with arachidonate) may not be confined to dynamin-dependent pinching-off of vesicles from donor membranes, but may be a common feature of vesicle formation and particularly membrane fission. Palmitoyl transfer to an unidentified acceptor is required in the pinching-off of Golgi-derived vesicles^{44–46}. Perhaps this acceptor, too, is LPA, given the observation that BARS-50, a protein substrate of brefeldin A-dependent ADP-ribosylation, induces the fission of Golgi tubules by palmitoyl transfer to a membrane lipid (R. Weigert *et al.*, unpublished observation). □

Methods

SLMV formation

SLMV formation in perforated PC12 cells, using cell-surface-biotinylated synaptophysin as marker (abbreviated as 'Biot. synaptophysin' in the Figures) and the single 30% glycerol step centrifugation to isolate SLMVs, was carried out and quantified as described²⁵. Differences in arbitrary units between Figure panels reflect differences in the exposure time of the synaptophysin immunoblots rather than in the efficiency of SLMV formation. In the standard control condition (cytosol containing 0.75 mg protein per ml), $0.8 \pm 0.2\%$ of the total biotinylated synaptophysin was recovered in SLMVs²⁵. To address the role of fatty acyl-CoA we used gel-filtered cytosol (PD-10 column, Pharmacia; GGA buffer) supplemented with 1 mM GTP. LPA (L- α -lysophosphatidic acid, oleoyl (C18:1, (cis)-9), Sigma) was added from a 10 mM stock in LPA buffer (20 mM Hepes-KOH pH 7.4, 10 mM sucrose, 1 mM EDTA, 2.5 mg ml⁻¹ fatty acid-free BSA (Boehringer)) sonicated (Sonorex super RK 102H) for 2 min; reactions without LPA contained the corresponding amount of LPA buffer. LPC (L- α -lysophosphatidylcholine, decanoyl (C10:0), Sigma), palmitoyl-CoA (Sigma) and arachidonoyl-CoA (Fluka) were added from 10 mM, 10 mM and 50 mM stocks in water, respectively. MAFF (Biomol Research Laboratories) was added from a 135 mM stock in DMSO; reactions without MAFF contained the corresponding amount of DMSO. PLA₂ from *Crotalus adamanteus* venom (CellSystems) was added from a 1 mg protein per ml stock in water. PLD from *Streptomyces chromofuscus* (Sigma) was added from a 100 U μl^{-1} stock in 10 mM Hepes-KOH pH 7.4 to a final concentration of 0.01–10 U ml⁻¹.

When investigating the role of arachidonoyl-CoA (Fig. 5b right), the pellet of perforated PC12 cells ($\sim 15 \text{ mg protein}$) was resuspended in 2 ml of the GGA/glutathione buffer²⁵ containing 10 mg ml⁻¹ fatty acid-free BSA, followed by addition of 50 $\mu\text{g ml}^{-1}$ PLA₂ from *C. adamanteus* and incubation at 37 °C for 15 min. All subsequent steps were performed at 4 °C. The BSA/PLA₂-treated perforated cells were washed and resuspended in GGA/glutathione buffer and used for perforated-cell reactions.

Purification of LPAAT from bovine brain

All steps were carried out at 4 °C. Bovine brains were homogenized with 3 volumes (v/v) of buffer A (50 mM Tris-HCl, pH 7.4; 250 mM sucrose; 2 mM EDTA; 50 μM PMSE; 0.1% (v/v) 2-mercaptoethanol) in a Waring Blender-type homogenizer. A particulate fraction containing most of the LPAAT activity present in the homogenate was obtained by differential centrifugation as follows: the homogenate for 10 min at 1,000g_{av}, the supernatant for 30 min at 5,000g_{av}, the supernatant for 2 h at 60,000g_{max}. The pellet was resuspended in buffer A to 15 mg protein per ml and diluted five-fold with buffer B (25 mM Hepes-NaOH pH 7.4, 2 mM EDTA, 4 mM CHAPS, 0.1% (v/v) 2-mercaptoethanol). Insoluble material was removed by centrifugation for 1 h at 100,000g_{max}; the resulting supernatant, referred to as Extract (Table 1), was loaded onto a Q-Sepharose column and LPAAT-activity was eluted with a 0–1.5 M NaCl-gradient in buffer C (50 mM Tris-HCl, pH 7.4; 250 mM sucrose; 2 mM EDTA; 2 mM CHAPS, 0.1% (v/v) 2-mercaptoethanol). The fractions of peak II were combined, dialysed against buffer D (50 mM potassium phosphate, pH 6.2, 2 mM EDTA; 0.1% (v/v) Nonidet P-40; 0.1% (v/v) 2-mercaptoethanol), loaded onto an S-Sepharose column and LPAAT was eluted with a linear 0–0.75 M NaCl gradient in buffer D. LPAAT-containing fractions were combined, dialysed against buffer E (20 mM Tris-HCl, pH 7.4; 10% (w/v) glycerol; 2 mM EDTA; 0.1% (v/v) Nonidet P-40; 0.1% (v/v) 2-mercaptoethanol) and loaded onto a Mono Q HR 10/10 column, and LPAAT was eluted with a 0–1 M NaCl gradient in buffer E. LPAAT-containing fractions were combined, dialysed against buffer F (20 mM Tris-HCl, pH 7.4; 1 mM EDTA; 10% (w/v) glycerol; 100 mM NaCl; 0.1% (v/v) Nonidet P-40; 10 μM PMSE; 0.1% (v/v) 2-mercaptoethanol) and chromatographed on a Superdex 75 Hiloal 16/60 column in buffer F. LPAAT-containing fractions were combined, concentrated using Centricon-30, loaded onto a Mono Q HR 5/10 anion-exchange column and LPAAT was eluted using a 0–1 M NaCl gradient in buffer E. LPAAT-containing fractions were concentrated using Centricon-30, diluted with one volume of 2x Laemmli sample buffer, heated for 2 min to 95 °C, loaded (300 μl) onto the cylindrical SDS-polyacrylamide gel column of a Model 491 Prep Cell (Biorad) electrophoresis apparatus and subjected to ESPACE according to the recommendations of the manufacturer. Fractions of 2 ml were collected and concentrated to $\sim 0.1 \text{ ml}$ using Centricon-30, and aliquots were analysed by analytical SDS-PAGE (Fig. 2a). Fractions containing the purified protein of $\sim 40\text{K}$ were diluted with buffer E (2 ml buffer E per 0.1 ml fraction) and concentrated to 0.1 ml by Centricon-30 centrifugation. This was repeated twice and, after this renaturation protocol, aliquots of the fractions were assayed for LPAAT activity (Fig. 2b). Fractions containing the $\sim 40\text{K}$ protein as the only detectable band and possessing LPAAT activity were combined and used for 2D-PAGE, sequence analysis and determination of substrate specificity.

Poly(L-proline) Sepharose adsorption and elution

All steps were performed at 4 °C. Rat brain cytosol²⁵ (1.5–3 ml, 7.5 mg protein per ml) was subjected to a single passage over a column containing ~1 ml of packed poly(L-proline)-coated Sepharose beads²⁸ and the flow-through was collected (depleted cytosol). The column was washed with 20 ml of GGA buffer²⁵ and eluted either directly with 2 ml of 8 M urea containing 10 mM Hepes-KOH, pH 7.4 or with 2 ml of 30% (w/v) DMSO diluted in GGA buffer followed by 2 ml of 8 M urea containing 10 mM Hepes-KOH, pH 7.4. The DMSO eluate (and, as control, 30% DMSO in GGA buffer) was extensively dialysed against GGA buffer containing 5 mM reduced glutathione, followed by concentration using Centricon-10 to ~200 µl, which contained ~30 µg of endophilin I as judged by SDS-PAGE using recombinant endophilin I as standard.

Protein identification

For MALDI, protein bands were excised from the gel and in-gel digested with trypsin as described⁴⁷. Peptide-mass mapping was performed on a Bruker Reflex mass spectrometer, and the list of peptide masses searched against a non-redundant protein sequence database with an accuracy better than 50 p.p.m.

For protein sequencing of tryptic peptides, ~10 µg of the 40K protein purified from bovine brain through the ESPACE step was digested twice for 2 h at 37 °C with 0.1 µg TPCK-trypsin. Peptides were separated by RP-HPLC (VYDAC 218 TP) using a 0.1% TFA/acetonitrile gradient and sequenced by the Edman method using an ABI sequencer.

Palmitoyl-CoA agarose adsorption and elution

All steps were performed at 4 °C. Rat brain cytosol (0.8 ml, 7.5 mg protein per ml) was subjected, with or without 40 mM added free palmitoyl-CoA, to a single passage over a column containing 1.5 ml of packed palmitoyl-CoA agarose beads (Sigma, equilibrated in GGA buffer), and the flow-through was collected. The column was washed with 20 ml of GGA buffer and eluted with 0.5 ml of GGA buffer containing 4 mM free palmitoyl-CoA (kept in the column overnight) followed by 3.5 ml of GGA buffer (total eluate 3.5 ml).

Recombinant endophilin I

A full-length endophilin I cDNA was generated by PCR (Pwo) using a mouse brain cDNA library and primers (sense primer 5'-CGAATACGAGGATCCATGTCGGTGG-CAGGGCTGAAG, antisense primer 5'-GCTTAACGAGAATTCCTAATGGGCGACAG-CAACCAG) designed according to the sequence of mouse endophilin I (ref. 18). The cDNA was cloned into pGEX2T (Pharmacia) and pQE30 (Qiagen) to obtain GST- and His₆-tagged fusion proteins, respectively.

A cDNA coding for a truncated endophilin I (amino acids 1–293, referred to as del-endophilin I), which lacks the SH3 domain, was generated by PCR using the full-length endophilin I cDNA, the above sense primer and the antisense primer 5'-TCGCGAAGCGGTACCGATCTGATCCATTGGACACCTGG, which encodes a stop codon after amino acid 293 and contains a *KpnI* restriction site. The PCR product was digested with *BamHI* and *KpnI* and ligated into pQE30 to obtain His₆-del-endophilin I.

The fusion proteins were expressed in BL21 or BL21-d (inducible for lysozyme) bacteria (Stratagene). Bacteria were lysed by two cycles of rapid freezing/thawing in PBS (25 ml per 1-L culture pellet). All subsequent steps were performed at 4 °C. After addition of aprotinin (5 µg ml⁻¹), leupeptin (10 µg ml⁻¹), benzamide (1 mM), PMSF (0.5 mM) and DNaseI (10 µg ml⁻¹, grade I, Boehringer) and incubation for 15 min, the lysate was centrifuged for 10 min at 10,000g and the resulting supernatant for 75 min at 280,000g. The high-speed supernatant (~6 mg protein per ml) was used for LPAAT assays (Fig. 3a), LPA-photoaffinity labelling (Fig. 4c), and the purification of recombinant endophilin I by either poly(L-proline) affinity chromatography (used to obtain the data shown in Figs 1c, 3b, c and 5b) or metal-chelate chromatography (used to obtain data not shown). In some experiments, pelleted bacteria were lysed (all steps at 4 °C) in 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazol, 2 mM Pefabloc (Boehringer) using sonication followed by addition of Triton X-100 (1% (v/v) final) and centrifugation for 15 min at 12,000g_{max}; the resulting supernatant was used for the purification of recombinant endophilin I using either metal-chelate chromatography (used to obtain the data shown in Figs 3d, 5, 6a, b, 7 and Table 2) or GSH affinity chromatography (used to obtain the data shown in Table 2).

Recombinant endophilin I was purified by poly(L-proline)-affinity chromatography as described above, except that DMSO was removed from the eluate by gel filtration using a PD-10 column equilibrated in GGA buffer followed by concentration using Centricon-10. Purification of recombinant endophilin I by metal-chelate chromatography was performed using Talon (Clontech) equilibrated with 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 10 mM imidazol, 2 mM Pefabloc; washing and elution used the same buffer except that imidazol was increased to 20 mM and 150 mM, respectively. Affinity chromatography using GSH-agarose was performed according to standard procedures. There was no obvious difference with regard to LPAAT activity and SLMV formation between the recombinant His₆-endophilin I purified by poly(L-proline) affinity chromatography from bacteria lysed by freezing/thawing, that purified by metal-chelate chromatography from such bacterial lysate, and that purified by metal-chelate chromatography from bacteria lysed by sonication/Triton X-100 (data not shown). As control, induced bacteria transformed with vector lacking the endophilin I insert were subjected in parallel to the same procedures of lysis, centrifugation and affinity chromatography, as indicated in the Figure legends.

Photoaffinity labelling using [³²P]1-(10-azido-stearoyl)-LPA

10-azido-stearic acid was prepared from 10-keto-stearic acid methyl ester (methyl 10-oxooctadecanoate, Aldrich). AtT-20 cells (one 10-cm dish) were grown for 16 h in 8 ml of

phosphate-free DMEM (Sigma) supplemented with 18% serum (horse serum/FCS 2/1, delipidated by extraction with organic solvent (C.T., Y. Wang & W.B.H., manuscript in preparation)) and containing ~70 µM 10-azido-stearic acid (solubilized in serum⁴⁸) and 1.5 mCi carrier-free inorganic [³²P]phosphate. The cells were extracted with chloroform/methanol and the chloroform phase subjected to thin-layer chromatography (TLC) using silica gel 60 (0.25 mm, Merck) and chloroform/methanol/water 65/35/8 as solvent. Phosphatidylcholine (PC, identified by minimal iodine vapour) was scraped from the plate, extracted using chloroform/methanol/water 65/35/8 and dried. After redissolving in 800 µl of diethylether/methanol 98/2, PC was converted to LPC using 250 µg PLA₂ (*C. adamanteus*) for 2 h at 25 °C (ref. 50). The whole reaction mixture was dried, redissolved in 500 µl 20 mM Tris-HCl pH 7.6, 5 mM CaCl₂, 1% Triton X-100, and after addition of PLD (1000 U, *S. chromofuscus*, Sigma) vigorously shaken at 30 °C for 2 h to convert LPC to LPA. After drying, lipids were dissolved in 300 µl chloroform/methanol/water 65/35/8 and subjected to TLC (chloroform/methanol/water/AcOH 65/35/8/6). The region of the thin-layer plate containing the [³²P]1-(10-azido-stearoyl)-LPA (identified by phosphoimaging and deduced from the mobility of LPA) was scraped and extracted with chloroform/methanol/water/AcOH 65/35/8/6. The yield of the final product was ~8 µCi.

For each photoaffinity-labelling reaction, 20 µl of [³²P]1-(10-azido-stearoyl)-LPA (10 µCi ml⁻¹, specific activity in the range of 0.1–1 µCi µg⁻¹) was dried and redissolved in 25 µl of 50 mM Hepes-NaOH pH 8.0 under sonication for 2 min. After addition of 50 µl of GGA buffer and 25 µl of 280,000g supernatant (150 µg protein) from a lysate of bacteria transformed without or with the insert coding for His₆-endophilin I, the samples were either placed under the filtered, focused beam (>310 nm) of a mercury lamp and UV-irradiated for 1 min at room temperature or kept for 1 min at room temperature without UV irradiation, followed in either case by addition of Laemmli sample buffer.

LPAAT assays

For the results shown in Fig. 2b and Tables 1 and 2, LPAAT activity was assayed in a final reaction volume of 200 µl containing, in addition to appropriate aliquots of the fractions obtained during and after purification, 10 mM Tris-HCl, pH 7.4; 0.1 mM EDTA, 25 mM sucrose; 0.05% (w/v) BSA; 100 µM of LPA (1-oleoyl) (or the other lyso-phospholipids indicated in Table 2); 40 µM [¹⁴C]oleoyl-CoA (10–15 µCi mol⁻¹). To determine the substrate specificity of purified and recombinant endophilin I, 7 µg, 2 µg and 10 µg of endophilin I purified from bovine brain, His₆-endophilin I, and GST-endophilin I, respectively, was used per assay. Reactions were carried out for 10 min at 37 °C and terminated by the addition of 2 ml chloroform/methanol (2/1), followed by addition of 1 ml 0.1 M KCl. The organic phase was collected, dried, and subjected to TLC using chloroform/acetone/methanol/acetic acid/water (50/20/10/10/5). Radioactivity in phosphatidic acid on the TLC plates was determined using a Berthold thin-layer radioactivity scanner.

For determining the effect of phosphorylation of endophilin I on its LPAAT activity, we incubated 2.2 µg of either endophilin I purified from bovine brain, His₆-endophilin I or GST-endophilin I for 30 min at 37 °C in a final reaction volume of 20 µl containing in addition 40 mM Hepes-NaOH pH 7.4, 9 mM MgCl₂, 0.23 mM ATP and 7.5 µg ml⁻¹ catalytic subunit of PKA. After the phosphorylation reaction, samples were subjected to LPAAT assay as above. The LPAAT activity observed after a phosphorylation reaction in the absence of added PKA was similar to that observed without performing the phosphorylation reaction.

For the results shown in Figs 3 and 7, aliquots of 1-[¹⁴C]arachidonoyl-CoA (75–750 nCi, 56 mCi per mmol, Moravsek Biochemicals) in ethanol/water (1:1) were dried in a SpeedVac and each dissolved in 150 µl of GGA buffer containing 5 mM reduced glutathione followed by sonication for 2 min. The tubes were then transferred to ice and brought to a final reaction volume of 300 µl by sequential additions as follows: (1) 137 µl or 122 µl of GGA buffer depending on the source of endophilin I to be added; (2) 3 µl of either the 10 mM LPA stock (see above), 1 mM LPA (made by 10-fold dilution of the 10 mM stock with LPA buffer followed by sonication for 4 min) or LPA buffer; (3) 10 µl of GGA buffer without or with ~3 µg of recombinant endophilin I, or 25 µl of the dialysed, concentrated and glycerol-diluted DMSO eluate without or with ~1.5 µg of endophilin I. Reactions were carried out for 15 min at 37 °C and terminated by addition of 300 µl ice-cold 0.8 M KCl, 0.2 M H₃PO₄. Samples were mixed with 900 µl chloroform/methanol (2:1) and centrifuged for 5 min at 10,000g. The lower phase was collected, dried, redissolved in 35 µl of chloroform/methanol/water (2:1:0.1), subjected (along with a [¹⁴C]-phosphatidic acid standard, NEN) to TLC using silica gel 60 and chloroform/pyridine/formic acid (50:30:7) as solvent, and analysed by phosphoimaging.

Miscellaneous

Recombinant human profilin I and mouse profilin II were obtained as described²⁷. Two-dimensional PAGE was performed using ampholytes pH 3–10 for isoelectric focusing. A rabbit antiserum specifically recognizing endophilin I was obtained from Eurogentec, using the synthetic peptide (AcNH-KPRMSLEFATGDGT-CONH₂), corresponding to residues 258–271 of rat endophilin I (ref. 30), coupled to KLH-Glu as antigen. Anti-Grb2 antibody was obtained from Transduction Laboratories.

Received 9 June; accepted 26 July 1999.

1. Matsuoka, K. *et al.* COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. *Cell* **93**, 263–275 (1998).
2. Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R. & Orci, L. Coatomer, ARF1p, and nucleotide are required to bud coat protein complex I-coated vesicles from large synthetic liposomes. *Proc. Natl Acad. Sci. USA* **95**, 11199–11204 (1998).
3. Bremser, M. *et al.* Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. *Cell* **96**, 495–506 (1999).
4. Takei, K., McPherson, P. S., Schmid, S. L. & De Camilli, P. Tubular membrane invaginations coated by

- dynamins are induced by GTP- γ S in nerve terminals. *Nature* **374**, 186–190 (1995).
5. Warnock, D. E. & Schmid, S. L. Dynamin GTPase, a force-generating molecular switch. *BioEssays* **18**, 885–893 (1996).
6. Cremona, O. & De Camilli, P. Synaptic vesicle endocytosis. *Curr. Opin. Neurobiol.* **7**, 323–330 (1997).
7. Schmid, S. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* **66**, 511–548 (1997).
8. Schmid, S. L., McNiven, M. A. & De Camilli, P. Dynamin and its partners: a progress report. *Curr. Opin. Cell Biol.* **10**, 504–512 (1998).
9. Hirst, J. & Robinson, M. S. Clathrin and adaptors. *Biochim. Biophys. Acta* **1404**, 173–193 (1998).
10. Vallee, R. B. & Okamoto, P. M. The regulation of endocytosis: identifying dynamin's binding partners. *Trends Cell Biol.* **5**, 43–47 (1995).
11. Hinshaw, J. E. & Schmid, S. L. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature* **374**, 190–192 (1995).
12. Sweitzer, S. M. & Hinshaw, J. E. Dynamin undergoes a GTP-dependent conformational change causing vesiculation. *Cell* **93**, 1021–1029 (1998).
13. Takei, K. *et al.* Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. *Cell* **94**, 131–141 (1998).
14. Stowell, M. H. B., Marks, B., Wigge, P. & McMahon, H. T. Nucleotide-dependent conformational changes in dynamin: evidence for a mechanochemical molecular spring. *Nature Cell Biol.* **1**, 27–32 (1999).
15. Roos, J. & Kelly, R. B. Is dynamin really a 'pinchase'? *Trends Cell Biol.* **7**, 257–259 (1997).
16. Sever, S., Muhlbarg, A. B. & Schmid, S. L. Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis. *Nature* **398**, 481–486 (1999).
17. Kirchhausen, T. Boa constrictor or rattlesnake? *Nature* **398**, 470–471 (1999).
18. Sparks, A. B., Hoffmann, N. G., McConnell, S. J., Fowlkes, D. M. & Kay, B. K. Cloning of ligand targets: systematic isolation of SH3 domain-containing proteins. *Nature Biotechnol.* **14**, 741–744 (1996).
19. Lipowsky, R. Domain-induced budding of fluid membranes. *Biophys. J.* **64**, 1133–1138 (1993).
20. Chernomordik, L., Kozlov, M. M. & Zimmerberg, J. Lipids in biological membrane fusion. *J. Membrane Biol.* **146**, 1–14 (1995).
21. Düzgünes, N. in *Trafficking of Intracellular Membranes* (eds Pedrosa de Lima, M. C., Düzgünes, N. & Hoekstra, D.) 97–129 (Springer, Heidelberg, 1995).
22. Chernomordik, L. V., Leikina, E., Frolov, V., Bronk, P. & Zimmerberg, J. An early stage of membrane fusion mediated by the low pH conformation of influenza hemagglutinin depends upon membrane lipids. *J. Cell Biol.* **136**, 81–93 (1997).
23. White, J. M. Membrane fusion. *Science* **258**, 917–924 (1992).
24. Kozlov, M. M. & Chernomordik, L. V. A mechanism of protein-mediated fusion: coupling between refolding of the Influenza hemagglutinin and lipid rearrangements. *Biophys. J.* **75**, 1384–1396 (1998).
25. Schmidt, A. & Huttner, W. B. Biogenesis of synaptic-like microvesicles in perforated PC12 cells. *Methods: A Companion to Methods Enzymol.* **16**, 160–169 (1998).
26. Schmidt, A., Hannah, M. J. & Huttner, W. B. Synaptic-like microvesicles of neuroendocrine cells originate from a novel compartment that is continuous with the plasma membrane and devoid of transferrin receptor. *J. Cell Biol.* **137**, 445–458 (1997).
27. Witke, W. *et al.* In mouse brain profilin I and II associate with regulators of the endocytic pathway and actin assembly. *EMBO J.* **17**, 967–976 (1998).
28. Janney, P. A. Polyproline affinity method for purification of platelet profilin and modification with pyrene-maleimide. *Methods Enzymol.* **196**, 92–99 (1991).
29. Micheva, K. D., Ramjaun, A. R., Kay, B. K. & McPherson, P. S. SH3 domain-dependent interactions of endophilin with amphiphysin. *FEBS Lett.* **414**, 308–312 (1997).
30. Ringstad, N., Nemoto, Y. & De Camilli, P. The SH3p4/SH3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. *Proc. Natl Acad. Sci. USA* **94**, 8569–8574 (1997).
31. De Heuvel, E. *et al.* Identification of the major synaptojanin-binding proteins in brain. *J. Biol. Chem.* **272**, 8710–8716 (1997).
32. Shupliakov, O. *et al.* Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. *Science* **276**, 259–263 (1997).
33. Desnos, C., Clift-O'Grady, L. & Kelly, R. B. Biogenesis of synaptic vesicles *in vitro*. *J. Cell Biol.* **130**, 1041–1049 (1995).
34. Söling, H.-D., Machado-De Menezes, E., Kleineke, J. & Fest, W. Early effects of β -adrenergic and muscarinic secretagogues on lipid and phospholipid metabolism in guinea pig parotid acinar cells. *J. Biol. Chem.* **262**, 16786–16792 (1987).
35. Söling, H.-D. *et al.* Mechanisms of short-term (second range) regulation of the activities of enzymes of lipid and phospholipid metabolism in secretory cells. *Adv. Enzyme Regul.* **28**, 35–50 (1989).
36. Söling, H.-D., Fest, W., Schmidt, T., Esselmann, H. & Bachmann, V. Signal transmission in exocrine cells is associated with rapid activity changes of acyltransferases and diacylglycerol kinase due to reversible protein phosphorylation. *J. Biol. Chem.* **264**, 10643–10648 (1989).
37. Coleman, J. Characterization of the *Escherichia coli* gene for 1-acyl-sn-glycerol-3-phosphate acyltransferase (plcC). *Mol. Gen. Genet.* **232**, 295–303 (1992).
38. Lio, Y. C., Reynolds, L. J., Balsinde, J. & Dennis, E. A. Irreversible inhibition of Ca^{2+} -independent phospholipase A2 by methyl arachidonyl fluorophosphonate. *Biochim. Biophys. Acta* **1302**, 55–60 (1996).
39. Eberhardt, C., Gray, P. W. & Tjoelker, L. W. Human lysophosphatidic acid acyltransferase: cDNA cloning, expression, and localization to chromosome 9q34.3. *J. Biol. Chem.* **272**, 20299–20305 (1997).
40. West, J. *et al.* Cloning and expression of two lysophosphatidic acid acyltransferase cDNAs that enhance cytokine-induced signaling responses in cells. *DNA Cell Biol.* **6**, 691–701 (1997).
41. De Camilli, P., Emr, S. D., McPherson, P. S. & Novick, P. Phosphoinositides as regulators in membrane traffic. *Science* **271**, 1533–1538 (1996).
42. Warnock, D. E. & Schmid, S. L. Dynamin GTPase, a force-generating molecular switch. *BioEssays* **18**, 885–893 (1996).
43. Tüscher, O., Lorra, C., Bouma, B., Wirtz, K. W. A. & Huttner, W. B. Cooperativity of phosphatidylinositol transfer protein and phospholipase D in secretory vesicle formation from the TGN—phosphoinositides as a common denominator? *FEBS Lett.* **419**, 271–275 (1997).
44. Glick, B. S. & Rothman, J. E. Possible role for fatty acyl-coenzyme A in intracellular protein transport. *Nature* **326**, 309–312 (1987).
45. Pfanner, N. *et al.* Fatty acyl-coenzyme A is required for budding of transport vesicles from Golgi cisternae. *Cell* **59**, 95–102 (1989).
46. Ostermann, J. *et al.* Stepwise assembly of functionally active transport vesicles. *Cell* **75**, 1015–1025 (1993).
47. Shevchenko, A. *et al.* Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl Acad. Sci. USA* **93**, 14440–14445 (1996).
48. Spector, A. A. & Hoak, J. C. An improved method for the addition of long-chain free fatty acid to protein solutions. *Anal. Biochem.* **32**, 297–302 (1969).
49. Morris, S. A. & Schmid, S. L. The Ferrari of endocytosis? *Curr. Biol.* **5**, 113–115 (1995).
50. Kates, M. *Techniques in Lipidology* (Elsevier, Amsterdam, 1986).

Acknowledgements

We thank L. Chernomordik, D. Corbeil, M. J. Hannah and I. Trompeter for advice and discussion; M. Fischer and D. Hesse for technical assistance; and A. Summerfield for artwork. M.W. was supported by predoctoral fellowships from the Friedrich-Ebert-Stiftung and the Graduiertenkolleg 'Molekularbiologische Analyse pathophysiologischer Prozesse'. W.B.H. and H.-D.S. were supported by grants from the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft.

Correspondence and requests for materials should be addressed to W.B.H. (e-mail: whuttner@sun0.urz.uni-heidelberg.de) or H.-D.S. (hsolin@gwdg.de).

Evidence of a supernova origin for the black hole in the system GRO J1655 – 40

G. Israelian*, R. Rebolo*†, G. Basri‡, J. Casares* & E. L. Martín*‡

* Instituto de Astrofísica de Canarias, E-38200 La Laguna, Tenerife, Spain

† Consejo Superior de Investigaciones Científicas, Spain

‡ Astronomy Department, University of California, Berkeley, California 94720, USA

Stars with masses greater than about ten solar masses are thought to end their lives either in a supernova¹ or in a direct gravitational collapse process², either of which could have a black hole as a remnant. But there is as yet no direct observational evidence to support either gravitational collapse in general or the formation of black hole remnants in particular. Here we report a large overabundance of oxygen, magnesium, silicon and sulphur in the atmosphere of the star orbiting a probable black hole in the binary system GRO J1655–40 (also known as Nova Scorpii 1994). These α -elements are six to ten times more abundant in the star's atmosphere than they are in the Sun's. We interpret these high abundances as evidence for supernova ejecta captured by the companion star. The relative abundances of these elements suggest that the supernova progenitor was in the mass range 25–40 solar masses.

We obtained a high-resolution spectrum of the secondary star in the eclipsing low-mass X-ray binary GRO J1655–40, in quiescence phase, on 24 May 1998 with the 10-m Keck I telescope. This system consists of a black hole with a mass estimated in the range 4.1–7.9 $M_{\odot}$ and a F3–F8 IV/III star of 1.6–3.1 $M_{\odot}$, orbiting the black hole at a distance of $\sim 17 R_{\odot}$ (refs 3–5); here $M_{\odot}$ and $R_{\odot}$ are respectively the mass and radius of the Sun. In Fig. 1 we plot the spectral range containing the most relevant lines for our analysis and also superimpose spectra of template stars of similar spectral type. In Table 1,

we provide information for these lines including measurements of equivalent widths (W_{λ}) from our spectrum, as well as average values from the spectra of the templates. First, we note the remarkable strength of the O I triplets and the Mg, Si and S lines in GRO J1655–40 as compared with the template, in contrast with the Fe lines, which are very similar in both. The comparison of the W_{λ} values measured in GRO J1655–40 with high-quality spectra of many other F-type standards⁶ provides additional confirmation that α -elements have stronger absorption lines in this otherwise normal F-type star.

In order to determine abundances from the observed spectrum and measured equivalent widths we have used well known model atmospheres⁷, the spectral synthesis code WITA (ref. 8) and atomic data from the VALD database⁹. We have modified the oscillator strengths of the lines from their original values until we succeeded in reproducing the solar atlases¹⁰ with solar abundances¹¹. As a check, the final set of oscillator strengths (Table 1) was then used to synthesize the spectrum of the F6 III standard star HR870. (This synthesis used values of $T_{\text{eff}} = 6,400$ K, $\log g = 3.7$, $[\text{Fe}/\text{H}] = 0.0$ and $\xi = 2 \text{ km s}^{-1}$ from available calibrations¹², where T_{eff} is effective temperature, $\log g$ is the logarithm of surface gravity, $[\text{Fe}/\text{H}]$ is a measure of the relative abundance of elements Fe and H with respect to the solar value (see Table 1 for details), and ξ is microturbulence.) According to recent work⁵, F6 III/F7 IV is the most likely spectral-type and luminosity classification of the secondary of GRO J1655–40; we therefore adopted this model atmosphere to conduct the abundance analysis. We have verified that a difference of one or two spectral subclasses does not imply a significant change in the final abundances.

In Fig. 1a and b we show the comparison between the synthetic and observed line profiles for different oxygen abundances. It is known that O I triplets are sensitive to non-local thermodynamic equilibrium (LTE) effects¹³. A non-LTE analysis using the synthesis code MULTI (ref. 14) and the oxygen model atom from the Pandora Atmosphere Program¹⁵ shows that an LTE analysis would overestimate oxygen abundance by 0.7 dex (dex is a difference in logarithmic values) and 0.4 dex for O I 7771–7775 Å and 8446 Å

Table 1 Chemical abundances for GRO J1655–40 secondary

λ (Å)	Ion	χ (eV)	$\log gf$	GRO J1655–40 W_{λ} (mÅ)	GRO J1655–40 [X/H]	Template* W_{λ} (mÅ)	Template* [X/H]
6,633.42	Fe I	4.83	–1.397				
6,633.75	Fe I	4.56	–0.698	105 $\pm$ 15	0.00 $\pm$ 0.10	110 $\pm$ 10	0.05 $\pm$ 0.10
6,634.12	Fe I	4.79	–1.420				
6,663.24	Fe I	4.56	–1.484	80 $\pm$ 15†	0.10 $\pm$ 0.15	90 $\pm$ 10	0.20 $\pm$ 0.10
6,663.44	Fe I	2.42	–2.958				
6,677.99	Fe I	2.69	–1.879	130 $\pm$ 20†	0.20 $\pm$ 0.15	120 $\pm$ 15	0.15 $\pm$ 0.10
6,749.03	Cr I	4.39	–1.075	80 $\pm$ 15†	0.15 $\pm$ 0.20	85 $\pm$ 15	0.20 $\pm$ 0.20
6,750.16	Fe I	2.42	–2.562				
7,780.56	Fe I	4.47	–0.474	90 $\pm$ 15	0.00 $\pm$ 0.20	95 $\pm$ 10	0.05 $\pm$ 0.15
7,771.95	O I	9.14	+0.284				
7,774.17	O I	9.14	+0.148	1,050 $\pm$ 80	1.30 $\pm$ 0.10‡	320 $\pm$ 30	–0.05 $\pm$ 0.15‡
7,775.39	O I	9.14	–0.144				
8,446.24	O I	9.52	–0.523				
8,446.35	O I	9.52	+0.170	530 $\pm$ 50	1.10 $\pm$ 0.10‡	250 $\pm$ 30	0.00 $\pm$ 0.15‡
8,446.75	O I	9.52	–0.050				
8,693.93	S I	7.87	–0.397	230 $\pm$ 25	0.90 $\pm$ 0.15	95 $\pm$ 15	–0.10 $\pm$ 0.15
8,694.62	S I	7.87	+0.176				
8,728.01	Si I	6.18	–0.263	250 $\pm$ 25	1.00 $\pm$ 0.25	130 $\pm$ 20	0.10 $\pm$ 0.20
8,728.59	Si I	6.18	–1.720				
8,736.02	Mg I	5.94	–0.154	200 $\pm$ 20	1.10 $\pm$ 0.40	115 $\pm$ 15	–0.25 $\pm$ 0.30
8,736.03	Mg I	5.94	–0.096				

Abundances (calculated assuming LTE) for elements are $[\text{X}/\text{H}] = \log [N(\text{X})/N(\text{H})]_{\text{star}} - \log [N(\text{X})/N(\text{H})]_{\text{sun}}$, where $N(\text{X})$ is number density of atoms. λ is the line wavelength, χ is the excitation energy of the lower level, gf is the oscillator strength and W_{λ} is the equivalent width of the blended feature. Errors are at the 1 σ level.

* Template values are obtained by averaging our measurements for stars HR870 (F7 IV), HR9057 (F8 III), HR6577 (F6 III), HR6985 (F5 III) and HR8392 (F4 III).

† Values obtained from the spectrum published by Shahbaz *et al.*⁵

‡ Values obtained from the non-LTE analysis.

triplets, respectively. In the spectrum of Fig. 1c there are clearly unblended absorption lines of Si, Mg and S, from which we derive the abundances given in Table 1, and more severely blended features from Ti and N which makes the abundance determination of these last elements rather uncertain. An enhancement in abundance of a factor of three appears to be required to fit the Ca II 866.2 nm line. However, this line is severely influenced by non-LTE and chromospheric effects and we warn that it may not be a good abundance indicator. The Fe lines in our star (Table 1) do not show any significant difference from those of the templates. We infer that the Fe abundance is almost solar, with $[\text{Fe}/\text{H}] = 0.1 \pm 0.2$, and, more generally, that the atmospheric structure of the star is very similar to that of normal F7/6 subgiant/giant stars. We fixed the abundance of Fe-group elements at the solar value and iterated the abundances of α -elements until the best fit to the spectra in Fig. 1 was achieved.

A large overabundance of α -elements as found in Table 1 may have an effect on the atmospheric structure and consequently α -element enhanced model atmospheres may be more suitable for the chemical analysis. We generated such models for fixed $[\alpha/\text{H}] = 1.0$ (the α -elements are O, Mg, Si, S, Ti and Ca) and repeated our analysis. We concluded that the abundances derived in Table 1 had to be reduced by 0.1–0.2 dex. After this correction our final values were $[\text{O}/\text{H}] = 1.0 \pm 0.3$, $[\text{S}/\text{H}] = 0.75 \pm 0.2$, $[\text{Mg}/\text{H}] = 0.9 \pm 0.40$, $[\text{Si}/\text{H}] = 0.9 \pm 0.3$, $[\text{Ti}/\text{H}] = 0.9 \pm 0.4$ and $[\text{N}/\text{H}] = 0.45 \pm 0.5$. The given 1σ errors take into account the uncertainties in the location of the continuum and in the determination of the stellar parameters ($\Delta T_{\text{eff}} = 250 \text{ K}$, $\Delta \log g = 0.3 \text{ dex}$). No signature of X-ray irradiation was found in our quiescence spectrum: the hydrogen lines were in absorption, Fe and some Fe-group elements (such as nickel and chromium) showed similar

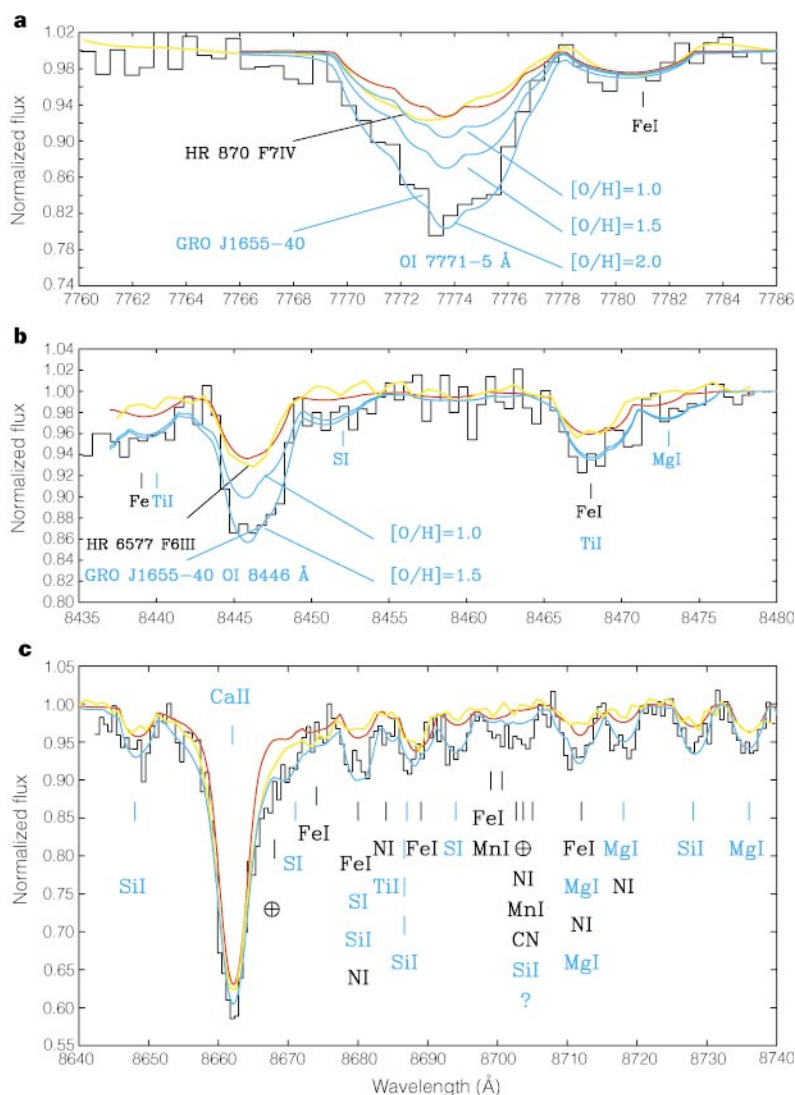


Figure 1 Spectral analysis of the secondary star in GRO J1655–40. The spectra of the secondary star (histograms) were obtained with the High Resolution Echelle Spectrograph²⁶ on the Keck 10-m telescope. The original dispersion and signal to noise ratio per pixel were 0.1 Å and 35, respectively. The spectra are binned in wavelength by a factor of five. Superimposed are several synthetic spectra (blue lines) computed in local thermal equilibrium for a range of oxygen abundances (**a** and **b**). α -elements are marked in blue. The best fit (blue line) to the target obtained for enhanced $[\alpha/\text{H}]$ ratios (see text) is shown in **c**. High-resolution spectra of two stars whose spectral types provide the best classification of the secondary star⁶ are shown (yellow lines) for comparison (**a**, HR870 F7

IV, obtained with the Hamilton echelle spectrograph at the 3-m Lick telescope; **b**, HR6577 F6 III, obtained from the literature⁶). Synthetic spectra computed for both templates adopting the stellar parameters described in the text and solar abundances are also shown (red lines). We did not make any effort to fit the broad absorption near 8,700 Å (indicated by blue question mark) because it is a complex blend of different lines and a telluric feature (crossed circle). The computed spectra and those of the templates have been broadened using a rotational profile to match the rotational broadening of the secondary. We derive a rotational velocity of $93 \pm 3 \text{ km s}^{-1}$ in good agreement with previous studies^{3,5}.

strengths in normal F5–F8 stars and no evidence was found for veiling from the disk. We can think of no physical mechanism that selectively acts on the spectral lines of the α -elements while the Fe-group, the hydrogen lines and the broad spectral energy distribution remain unaffected.

The secondary star clearly does not have sufficient mass to reach the internal temperatures needed to create these α -elements, but more massive stars can efficiently produce them in their interiors^{16–18}. In principle, nuclei produced during the hydrostatic burning phase of the massive progenitor of the black hole may have contaminated the companion via a stellar wind or mass transfer. Nevertheless, this contamination could supply only a small fraction of the currently observed abundance because the nuclear reactions in this evolutionary phase would have produced a much larger overabundance of nitrogen which is not seen¹⁹. Moreover, observations of very massive stars do not imply that their atmospheres are enriched by sulphur or silicon nuclei, thereby ruling out the possibility that a stellar wind may have contaminated the companion with such products.

The most natural way to expose the companion atmosphere to matter synthesized in the inner layers of the progenitor is through a supernova explosion, which must also account for both the chemical anomalies in the companion and the formation of a massive black hole. The latter requires a He core in the progenitor star with a minimum mass of 5–7 $M_{\odot}$; its maximum mass can be constrained using dynamical arguments. In a spherically symmetrical supernova explosion, the remnant binary will not be disrupted if the mass of the secondary is larger than the difference between the mass of the He core of the progenitor and twice the mass of the black hole²⁰. Thus, the maximum mass of the He core which would allow preservation of the binary is in the range 10–16 $M_{\odot}$. Current models^{18,21} for the evolution of a progenitor star of 25–40 $M_{\odot}$ propose the formation of He cores in the permitted mass range, their explosion as type II or type Ib supernovae ejecting at least 1–2 $M_{\odot}$ of matter enriched with nucleosynthetic products (such as O, S, Si and Mg), and the formation of black holes¹⁷ in the lower mass range estimated for the compact object in GRO J1655–40. Most of the Fe, and Fe group elements, synthesized during the explosion in the deeper layers of the progenitor, will go into the formation of the black hole itself, and consequently the ejecta will not be enriched in these elements. Our finding that the Fe abundance is not enhanced in the companion star strongly supports this case. Models of explosive nucleosynthesis for a 40 $M_{\odot}$ case show that the layers above those going into a 4 $M_{\odot}$ black hole will be ejected, containing O, S, Si and Mg in proportions similar to those seen in the companion star. If such a massive progenitor had a large angular momentum and a magnetic field possibly associated with the spiralling-in of the companion star, the collapse of the massive Fe core could lead to the formation of even more massive black holes and induce a ‘hypernova’²² explosion with nucleosynthesis yields²³ which are also consistent with our observations.

Given the solid angle subtended from the black hole³ and assuming that the ejected matter is distributed symmetrically around the compact object, about 10^{-3} $M_{\odot}$ (70% of which is oxygen¹⁸) could have been intercepted by the secondary, penetrated deep into the atmosphere and mixed with matter in the convective zone²⁴ ($\sim 10^{-4}$ $M_{\odot}$). Undermining the possibility that a companion star would be ablated by a nearby supernova, our observations imply that a fraction of the ejecta was in some way deposited and retained by the secondary, drastically altering the atmospheric α -element content. The apparent preservation of these atmospheric abundance anomalies implies that the secondary has not transferred a significant fraction of its mass to the compact object, and thus that the black hole was created with approximately its present mass. Given the estimated average accretion rate on to the black hole²⁵ of 1.3×10^{-10} $M_{\odot} \text{ yr}^{-1}$, and the mass in the convective zone, we also infer that less than 10^6 years have elapsed since the supernova.

Current models for stellar collapse after the supernova explosion¹⁷ suggest the formation of massive black holes after a short intermediate stage in which a neutron star is initially formed. A few seconds to several hours later, this neutron star accretes matter that, due to hydrodynamical interactions in the ejecta, is pushed back on to this star and forms the black hole. These models, when applied to our system, also help to explain the measured kick velocity⁵ of $141 \pm 1.3 \text{ km s}^{-1}$, which is rather high compared with other low-mass X-ray binaries containing black holes. It is known that neutron stars usually receive much higher kick velocities than that seen in the Nova Scorpii 1994 black-hole binary. If significant fall-back occurs²⁰ the kick velocity that the compact object receives is likely to be somewhat less than if there were no fall-back. To form the black hole in Nova Scorpii 1994 with at least 4 $M_{\odot}$, a significant fall-back probably took place which would have reduced the original kick to its present value. A fall-back of 3 $M_{\odot}$ after the supernova explosion is required in order to explain the kinematics of the system²⁰. This fall-back mass is perfectly plausible for a He core in the mass range 10–16 $M_{\odot}$. □

Received 26 January; accepted 23 July 1999.

- Maeder, A. Stellar yields as a function of initial metallicity and mass limit for black-hole formation. *Astron. Astrophys.* **264**, 105–120 (1992).
- Brown, G. E., Lee, C.-H. & Bethe, H. A. Evolution of black holes in the galaxy. *New Astron.* (in the press).
- Orosz, J. A. & Bailyn, C. D. Optical observations of GRO J1655–40 in quiescence. I. A precise mass for the black hole primary. *Astrophys. J.* **477**, 876–896 (1997).
- van der Hooft, F., Heemskerk, M. H. M., Alberts, F. & van Paradijs, J. The quiescence optical light curve of Nova Scorpii 1994 (= GRO J1655–40). *Astron. Astrophys.* **329**, 538–550 (1998).
- Shahbaz, T., van der Hooft, F., Casares, J., Charles, P. A. & van Paradijs, J. The mass of X-ray Nova Scorpii 1994 (= GRO J1655–40). *Mon. Not. R. Astron. Soc.* **306**, 89–94 (1999).
- Andrillat, Y., Jaschek, C. & Jaschek, M. Atlas of the infrared spectral region. The early type stars (O–GO). *Astron. Astrophys. Suppl.* **112**, 475–493 (1995).
- Kurucz, R. L. ATLAS9 Stellar Atmospheres Programs and 2 km s^{−1} Grid. (CD-ROM, Smithsonian Astrophysical Observatory, Cambridge, 1993).
- Pavlenko, Ya. V. Determination of C, N and O abundances in the atmospheres of late type stars—extreme value problem. *Sov. Astron.* **35**, 212–231 (1991).
- Piskunov, N. E., Kupka, E., Ryabchikova, T. A., Weiss, W. W. & Jeffery, C. S. VALD: The Vienna atomic line data base. *Astron. Astrophys. Suppl.* **112**, 525–535 (1995).
- Kurucz, R. L., Furenlid, I., Brault, J. & Testerman, L. Solar flux atlas from 296 to 1300 nm. *NOAO Atlas 1*, (Harvard Univ. Press, Cambridge, 1984).
- Anders, E. & Grevesse, N. Abundances of the elements—meteoritic and solar. *Geochim. Cosmochim. Acta* **53**, 197–214 (1989).
- Straizys, V. & Kurilina, G. Fundamental stellar parameters derived from the evolutionary tracks. *Astrophys. Space Sci.* **80**, 353–368 (1981).
- Kiselman, D. The 777 nm oxygen triplet in the sun and solar-type stars, and its use for abundance analysis. *Astron. Astrophys.* **275**, 269–282 (1993).
- Carlsson, M. A Computer Program for Solving Multi-Level Non-LTE Problems in Moving or Static Atmospheres (Rep. 33, Uppsala Astronomical Observatory, Sweden, 1986).
- Avrett, E. H. & Loeser, R. The Pandora Atmosphere Program. *Bull. Am. Astron. Soc.* **28**, 882 (1996).
- Arnett, W. D. *Nucleosynthesis and Supernovae* (Princeton Univ. Press, 1996).
- Woosley, S. E. & Weaver, T. A. The evolution and explosion of massive stars. II. Explosive hydrodynamics and nucleosynthesis. *Astrophys. J. Suppl.* **101**, 181–235 (1995).
- Thielemann, F.-K., Nomoto, K. & Hashimoto, M.-A. Core-collapse supernovae and their ejecta. *Astrophys. J.* **460**, 408–436 (1996).
- Langer, N. Wolf-Rayet stars of type WN/WC and mixing processes during core helium burning of massive stars. *Astron. Astrophys.* **248**, 531–537 (1991).
- Brandt, N., Podsiadlowski, Ph. & Sigurdsson, S. On the high space velocity of X-ray Nova Sco 1994: Implications for the formation of its black hole. *Mon. Not. R. Astron. Soc.* **277**, L35–L40 (1995).
- Nomoto, K. et al. Nucleosynthesis in Type II supernovae. *Nucl. Phys. A* **616**, 79c–90c (1997).
- Paczynski, B. Are gamma-ray bursts in star-forming regions? *Astrophys. J.* **494**, L45–L49 (1999).
- Nomoto, K., Nakamura, T., Iwamoto, K., Umeda, H. & Mazzali, A. in *Nuclei in the Cosmos V* (eds Prantzos, N. & Harissopoulus, S.) 252–258 (Editions Frontieres, Paris, 1999).
- D’Antona, F. & Mazzitelli, I. New pre-main sequence tracks for $M \leq 2.5 M_{\odot}$ as tests of opacities and convection model. *Astrophys. J. Suppl.* **90**, 467–500 (1994).
- van Paradijs, J. On the accretion instability in soft X-ray transients. *Astrophys. J.* **464**, L139–L141 (1996).
- Vogt, S. et al. HIREs: the high-resolution echelle spectrometer on the Keck 10m telescope. *Proc. SPIE* **2198**, 362–375 (1994).

Acknowledgements

We thank Y. Pavlenko for providing model atmospheres with α -element enhancement and H. Socas for helping with non-LTE computations of the O I triplet. The data presented here were obtained at the W. M. Keck Observatory, which is operated as a scientific partnership among the California Institute of Technology, the University of California and NASA. The Observatory was made possible by the financial support of the W. M. Keck foundation.

Correspondence and requests for materials should be addressed to R.R. (e-mail: rrl@ll.iac.es).

Charge fluctuations in $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ high-temperature superconductors

H. A. Mook* & F. Doğan†

* Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6393, USA

† Department of Materials Sciences and Engineering, University of Washington, Seattle, Washington 98195, USA

Understanding the behaviour of the electrons in the high-temperature copper oxide superconductors remains a challenging problem. An important class of models^{1–5} argues that the distribution of electronic charge and spin is not homogeneous: rather, spin and charge adopt a dynamic arrangement in which the spins on the copper form antiferromagnetic stripes, separated by domain walls containing the charge^{1–5}. The dynamic behaviour of the spins has been extensively studied by neutron scattering, and recent results⁶ have shown that the low-frequency fluctuations for different classes of materials display a universal spatial behaviour that is consistent with the stripe picture. But arguments for the existence of the stripe phases are difficult to sustain without a demonstration that charge is distributed in the domain walls. Here we report phonon measurements for the $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ high-temperature superconductors, which reveal the presence of charge fluctuations. The inferred periodicity is that expected if the charge is located in the domain walls separating the spin stripes. Our results therefore provide strong support for the existence of a dynamic stripe phase in the high-temperature superconductors.

To decide whether the stripe-phase picture is appropriate for the copper oxide superconductors, it is necessary to determine the spatial arrangement of the charge and spin. As neutrons have no charge, they cannot interact directly with either static or dynamic charge structure in materials. However, neutrons can be used to observe charge structure indirectly by measuring the atomic displacements that result from the charge distribution. Static and dynamic spin structure can be observed by the direct interaction between the neutron spin and the electron spin, and detailed information is much easier to obtain for spin structure than for charge structure. Because extensive information is available on the spin structure of the copper oxide superconductors, we consider spin first to give an indication of what might be expected for charge structure.

The parent compounds of the high- T_c materials are insulating antiferromagnets that display a magnetic unit cell doubling resulting in a lowest-order magnetic peak with a wavevector of $(1/2, 1/2)$ (refs 7, 8). Here we use reciprocal lattice units (r.l.u.) to label wavevectors in two-dimensional reciprocal space such that $(1,0)$ and $(0,1)$ are along the nearest-neighbour copper–oxygen–copper paths and are the locations of the lowest-order diffraction peaks from the nuclei in the nearly square CuO_2 planes (Figs 1a, b). Chemical doping is used to introduce charge into the insulator, resulting in metallic and superconducting behaviour. In this case the static antiferromagnetic order usually disappears, being replaced by strong antiferromagnetic fluctuations that can be measured with inelastic neutron spectroscopy. It is now well established that for superconducting compositions the magnetic fluctuations are found at incommensurate positions^{6,9–11} in the reciprocal lattice $\delta/2$ from the $(1/2, 1/2)$ position as shown in Fig. 1b; δ is related to the number of charges (holes) introduced into the Cu–O planes¹². The incommensurate peaks can be considered to result from the spin stripes of a dynamic striped phase^{1–5}, or to be derived from spin flip scattering across a nested Fermi surface^{13–15}.

The stripe-phase interpretation gained much prominence from studies by Tranquada *et al.*^{16,17} on the low- T_c material $\text{La}_{1.88-y}\text{Nd}_y\text{Sr}_{0.12}\text{CuO}_4$. These studies showed that, for $y = 0.4$, an elastic magnetic component or spin density wave (SDW) produces

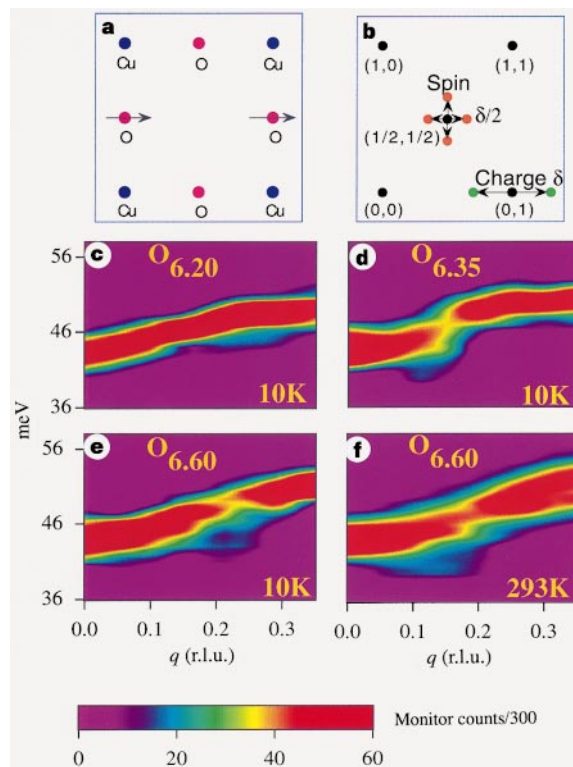


Figure 1 Phonon-intensity measurements for the $(B_{2u}B_{3u})$ planar oxygen mode in YBCO_{7-x} for three oxygen compositions. **a**, Arrangement of Cu and O in the Cu–O planes of the copper oxide superconductors where the small orthorhombic distortion has been neglected. The arrows show the motions of the atoms for the phonon mode investigated. **b**, Reciprocal lattice diagram showing the locations of spin and charge. The incommensurate spin fluctuations are located at the end of the arrows surrounding the magnetic superlattice position $(1/2, 1/2)$. Charge fluctuations are observed at the locations shown at the end of the arrows surrounding a reciprocal lattice position: the $(1,0)$ position is shown as an example. **c–f**, Phonon measurements for the $(B_{2u}B_{3u})$ branch plotted in units of energy (meV) versus momentum q in reciprocal lattice units (r.l.u.). Small peaks from a different mode were subtracted on the high-energy side from scans with $q > 0.25$ (not in the region of interest) to give a clean picture of the $(B_{2u}B_{3u})$ mode. A background of 32 counts for the data obtained at 10 K and 50 counts for the 293 K data was determined from the average in the region between 55 and 57 meV and $q = 0–0.1$ r.l.u., and subtracted from the measurements to produce the data shown. The phonon structure factor $R(\omega)$ was found not to vary greatly over the q -range studied, but the integrated intensity at each q was normalized to the $q = 0$ value to give a unified picture of the phonon branch. Linear interpolation was then used to obtain the image of the phonon intensity on the contour maps. The colour bar is in units of 300 monitor counts which takes about 5 min at 45 meV. Multiple scans were averaged to decrease the statistical error.

peaks around the $(1/2, 1/2)$ position as in Fig. 1b at the same place where incommensurate fluctuations are observed with no Nd. This means that spin and charge could be studied by standard diffraction, rather than by more difficult inelastic scattering techniques. Incommensurate magnetic elastic or quasielastic peaks have also recently been found in $\text{La}_{2-x}\text{Sr}_x\text{Cu}_{1-y}\text{Zn}_y\text{O}_4$ (ref. 18) and in $\text{La}_2\text{CuO}_{4+\delta}$ (ref. 19). For the Nd-doped material, charge peaks—that can be considered to stem from charge density wave (CDW) ordering—were observed, split about the high-intensity nuclear Bragg peaks with a wavevector magnitude δ , as in Fig. 1b. The charge ordering has been confirmed by diffraction studies²⁰ using hard X-rays. Tranquada *et al.*^{16,17} interpreted the results as evidence for a static striped phase, by analogy with similar results found for non-superconducting $\text{La}_{2-x}\text{Sr}_x\text{NiO}_4$ (ref. 21) and because the CDW persisted to higher temperatures than the SDW. The two-to-one ratio between the wavevector of the CDW and the SDW is the result of a change in the antiferromagnetic phase of the stripe at the charge-domain boundary, resulting in a spin periodicity twice that of the charge periodicity.

No static spin structure has been observed in $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ (YBCO_{7-x}) high- T_c superconducting materials, and our experiments have also failed to find static charge order. To simplify notation, we can consider the incommensurate magnetic fluctuations that have been observed to be a dynamic SDW. The question then is whether there is a dynamic CDW associated with the established dynamic SDW that would signal the existence of a dynamic striped phase. The dynamic CDW cannot be observed directly by neutrons, but it is known that a CDW or a dynamic CDW can produce a pronounced anomaly in phonon dispersion curves at the CDW or dynamic CDW wavevector. (An example of this for TaSe_2 and NbSe_2 is discussed in ref. 22.) We expect a similar result for a dynamic CDW in the copper oxide superconductors, and thus have made detailed measurements of phonon lineshapes in YBCO_{7-x} crystals for compositions which have a dynamic SDW, in order to image any associated dynamic CDW that may be present.

Extensive phonon measurements in YBCO_{7-x} have been made by Pintschovius and Reichardt^{23,24}. However, their measurements concentrated on compositions with x near either one or zero, and few temperature-dependent results were obtained. We have repeated a number of their measurements and in general very good agreement has been found. Wavevector-dependent line widths for c -axis polarized phonons in YBCO_{7-x} (for x near zero) have been found by Reznik *et al.*²⁵ and Pyka *et al.*²⁶ but no connection was made to incommensurate spin structure. McQueeney *et al.*²⁷ have recently observed anomalous phonon dispersion in $\text{La}_{1.85}\text{Sr}_{0.15}\text{CuO}_4$, and suggested that this resulted from dynamic short-range unit-cell doubling. The type of charge ordering suggested by these authors is different from the stripe ordering found in $\text{La}_{1.48}\text{Nd}_{0.4}\text{Sr}_{0.12}\text{CuO}_4$, and as only one composition was studied, a direct connection between charge and spin fluctuations could not be established.

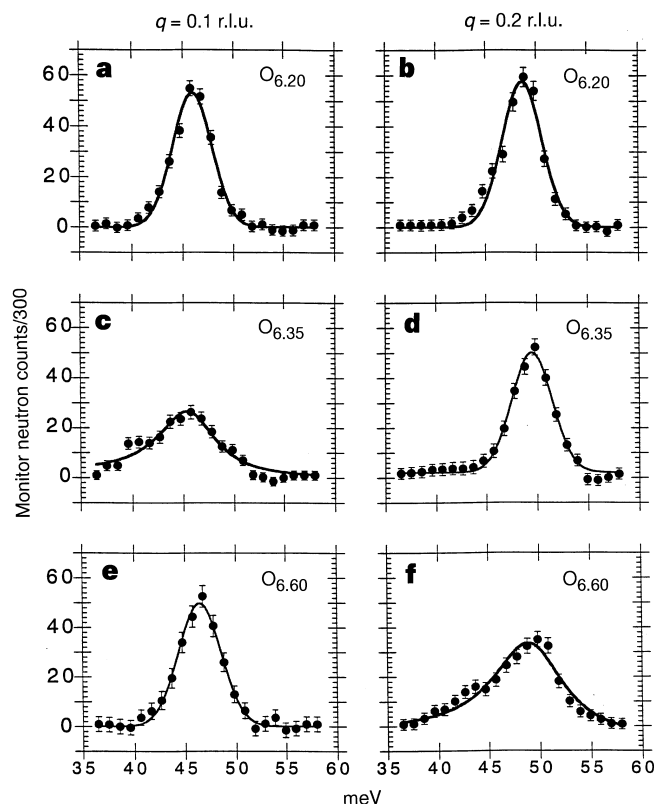


Figure 2 Energy scans (meV) obtained at values of q where substantial phonon broadening occurs in $\text{YBCO}_{6.35}$ and $\text{YBCO}_{6.60}$. **a, b**, $\text{YBCO}_{6.20}$; the line-widths are resolution-limited in this case. **c, d**, $\text{YBCO}_{6.35}$; a broad asymmetric line-width is found at $q = 0.1$ r.l.u., while a resolution-limited line-width is observed for $q = 0.2$ r.l.u. **e, f**, $\text{YBCO}_{6.60}$; the line-width for $q = 0.1$ r.l.u. may be slightly broader than resolution, but the width at $q = 0.2$ r.l.u. is asymmetric and considerably broadened.

We have searched for a dynamic CDW in YBCO_{7-x} by making measurements of phonon energy line shapes for materials where the wavevector of the dynamic SDW is known. A number of phonon branches were investigated, but because they are generally closely spaced in energy it is difficult to isolate a single branch. We eventually concentrated on the longitudinal branch identified by Pintschovius and Reichardt^{23,24} as the $(\text{B}_{2u}\text{B}_{3u})$ mode, as this branch could be well isolated and because it showed substantial effects. The atomic motions for this mode are mostly confined to the planar oxygens, with displacements occurring along the $a(b)$ direction as shown in Fig. 1a. The oxygen motions in the YBCO bilayers are in phase for this mode.

The measurements were made on the HB-2 and HB-3 triple-axis spectrometers at the High-Flux Isotope Reactor (HFIR) in the Oak Ridge National Laboratory. Most of the measurements were made with a vertically focused Be monochromator and a Si analyser using the (1,0,1) and (1,1,1) reflections, respectively. The spectrometers were operated with a fixed-analyser energy of either 30.5 or 13.7 meV. Collimation used for the 13.7 meV analyser energy was 48-40-60-120 min (full-width at half-maximum) from before the monochromator until after the analyser. Some measurements were made with a pyrolytic graphite analyser, but in this case great care had to be taken to avoid second-order scattering.

Crystals with three different oxygen concentrations were used in the measurements. One of the crystals was the same 25.6-g $\text{YBCO}_{6.60}$ sample ($T_c = 62.7$ K) that was used earlier for the magnetic measurements⁶. In this case, dynamic SDW peaks measured at an energy transfer of 24 meV are located at $\delta/2 = 0.105 \pm 0.01$ r.l.u. Measurements at 34 meV showed a commensurate resonance excitation which was sufficiently intense to overwhelm incommensurate scattering of the same magnitude as that found at 24 meV. The second superconducting sample had a composition of $\text{YBCO}_{6.35}$. A broad distribution was found for T_c with the onset being near 40 K. It is difficult to make samples with a sharply defined T_c with oxygen concentrations near 6.35, as T_c changes rapidly with small oxygen changes. Nevertheless, the sample had well-defined dynamic SDW peaks that were observed by triple-axis spectrometry at the HFIR. $\delta/2$ was observed to be 0.063 ± 0.01 , 0.057 ± 0.01 , 0.061 ± 0.01 and 0.055 ± 0.01 r.l.u. at energy transfers of 9, 15, 30 and 45 meV respectively. A commensurate resonance found at 23 meV dominated the scattering near this energy transfer. The scattering was thus found to be incommensurate above and below the resonance over an

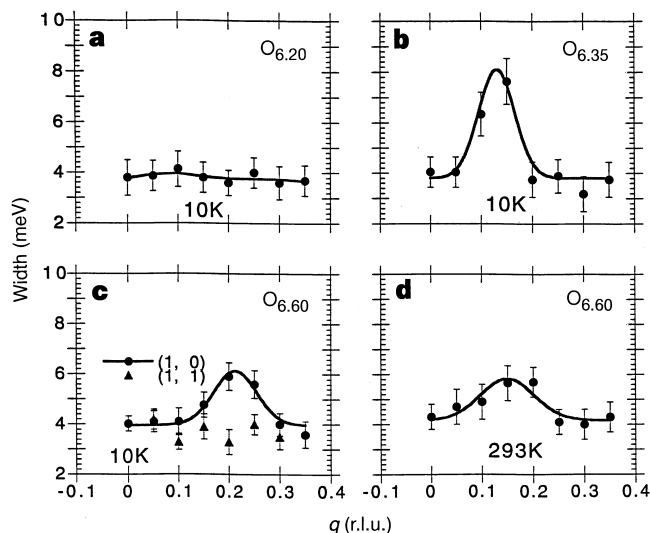


Figure 3 Phonon line-widths obtained from the least-squares fits of the phonon intensity ($I(\omega)$) convoluted with the spectrometer resolution. **a**, Widths for $\text{YBCO}_{6.20}$ are consistent with the resolution-limited value of 3.8 meV. **b-d**, Large line-widths occur at the values of q associated with charge fluctuations, for the (1,0) direction. No noticeable broadening is found for the (1,1) direction in $\text{YBCO}_{6.6}$.

extended energy range with a $\delta/2$ equal to 0.06 r.l.u., within the error of the measurements. These results on the magnetic response are part of a detailed study of the incommensurate and resonance scattering in YBCO (P. Dai, H.A.M. and F.D., manuscript in preparation). The third sample, YBCO_{6.20}, was an antiferromagnetic insulator with commensurate magnetic order and fluctuations. The three samples were essentially identical except for the oxygen composition.

Measurements of the $(B_{2u}B_{3u})$ mode were made using the standard triple-axis technique along the [1,0] direction around the (4,0,0) reflection. Clean constant momentum transfer (q) scans of the phonon peaks were obtained between (4,0,0) and (4.25,0,0). From (4.25,0,0) to (4.35,0,0) an additional mode is weakly observed at the high-energy end of the scan. Contour maps of the phonon measurements for the three samples are shown in Fig. 1c–f.

Figure 1c shows that the insulating YBCO_{6.20} phonon dispersion curve shows normal behaviour, and is resolution-limited in energy within the errors of the measurement. Figure 1d shows that the dispersion curve for YBCO_{6.35} acquires an extensive broadening of the line shape near $q = 0.1$ r.l.u. (that is, at about twice the wavevector of the dynamic SDW). Figure 1e shows an anomalous line shape for YBCO_{6.60}, found for q values near 0.2 r.l.u., which again is at twice the wavevector of the dynamic SDW for this material. In Fig. 1f a measurement is shown for the YBCO_{6.60} sample at 293 K. The phonon broadening remains, but appears to be more diffuse and to occur at somewhat lower values of q .

Phonon data for $q = 0.1$ and 0.2 r.l.u. are shown in Fig. 2. The phonon intensity distribution as a function of energy is given by the cross-section for phonon scattering multiplied by an assumed line shape $F(\omega)$. The intensity is then given simply by $I(\omega) = CR(\omega)F(\omega)n(\omega)/\omega$ where C is a constant, $R(\omega)$ is the dynamic structure factor, and $n(\omega)$ is the Bose population factor. We have assumed a lorentzian shape for $F(\omega)$, and the curves through the data points are a least-squares fit of $I(\omega)$ convolved with the spectrometer resolution (~ 3.8 meV) which is assumed to be gaussian. This results in a line shape that is nearly symmetric if the width is small, but is asymmetric with a low-energy tail for large widths. Line widths for YBCO_{6.20} in Fig. 2a and b are consistent with resolution-limited shapes. Figures 2c and d show that YBCO_{6.35} acquires a broad asymmetric line shape near $q = 0.1$ r.l.u., while Fig. 2e and f for YBCO_{6.60} shows extensive broadening at 0.2 r.l.u. Widths for phonons obtained from the least-squares fit are shown in Fig. 3. The large line widths for YBCO_{6.35} near $q = 0.1$ r.l.u., and for YBCO_{6.60} near $q = 0.2$ r.l.u., are apparent. We have also measured the phonon branch for the (1,1) direction for YBCO_{6.60}, and the widths are shown in Fig. 3c. No sizeable effect is observed for this direction, showing the similarity in the wavevector pattern for the charge and spin scattering.

The results show that the phonon branch for materials with a dynamic SDW has very distinct anomalies found at wavevectors twice that of the dynamic SDW. The anomalies are substantial, resulting in phonon broadening that is 10% of the phonon energy. It is a straightforward interpretation to relate the anomalies to a dynamic CDW. The atomic motions of the branch are the planar oxygens, and their light weight would probably make them acquire large displacements in response to the charge fluctuations. A logical assumption is that the experiments are showing charge dynamics for the high- T_c materials that are directly related to the static charge displacements found when T_c is reduced by the addition of Nd in La_{1.88}–_yNd_ySr_{0.12}CuO₄ (refs 16, 17). Accepting the stripe explanation for that system would then imply that dynamic striped phases are present in the copper oxide superconductors. Zachar *et al.*²⁸ considered the cases where a coupled CDW and SDW stem from either a nested Fermi surface or a striped phase. For the nested Fermi surface case, the system is spin-driven with charge following. We see from Fig. 1f and Fig. 3d that the charge fluctuations are still visible at 293 K for YBCO_{6.60}, although they appear to be less well-defined and shift to a somewhat lower q . Dai *et al.*²⁹ have shown that the incommensurate spin fluctuations are not observed above 150 K for YBCO_{6.60}. Our

results suggest that, for YBCO_{7–x}, charge is the driving force with spin following at reduced temperatures. In addition, the largest phonon anomalies are found for the YBCO_{6.35} sample which has a composition very near the antiferromagnetic boundary. We expect the Fermi surface to be less well-defined at this low doping level. The present results thus favour a stripe-phase interpretation.

The measurements that we report here show (we believe for the first time) that there are remarkable phonon anomalies in YBCO_{7–x} whose wavevector δ scales with oxygen content, and thus with hole doping. Furthermore, δ is twice the wavevector that is found for the spin fluctuations, showing a direct relationship between charge and spin fluctuations. These observations provide the essential elements needed for the existence of a dynamic striped phase in the high-temperature superconductors. The existence of such a phase would have a significant effect on our understanding of the superconducting process in these materials. □

Received 26 February; accepted 15 June 1999.

- Schulz, H. J. Domain walls in a doped antiferromagnet. *J. Phys. (Paris)* **50**, 2833–2849 (1989).
- Zaanen, J. & Gunnarson, O. Charged magnetic domain lines and the magnetism of high- T_c oxides. *Phys. Rev. B* **40**, 7391–7394 (1989).
- Poiblanc, D. & Rice, T. M. Charged solitons in the Hartree-Fock approximation to the large-U Hubbard model. *Phys. Rev. B* **39**, 9749–9752 (1989).
- Kivelson, S. A. & Emery, V. J. in *Strongly Correlated Electronic Materials: The Los Alamos Symposium 1993* (eds Bedell, K. S. *et al.*) 619–656 (Addison-Wesley, Maine, 1994).
- Emery, V. J. & Kivelson, S. A. Collective charge transport in high-temperature superconductors. *Physica C* **235–240**, 189–192 (1994).
- Mook, H. A. *et al.* Spin fluctuations in YBa₂Cu₃O_{6.6}. *Nature* **395**, 580–582 (1998).
- Vaknin, D. *et al.* Antiferromagnetism in La₂CuO_{4–x}. *Phys. Rev. Lett.* **58**, 2802–2805 (1987).
- Tranquada, J. M. *et al.* Neutron-diffraction determination of antiferromagnetic structure of Cu ions in YBa₂Cu₃O_{6+x} with $x = 0.0$ and 0.15. *Phys. Rev. Lett.* **60**, 156–159 (1988).
- Cheong, S.-W. *et al.* Incommensurate magnetic fluctuations in La_{2–x}Sr_xCuO₄. *Phys. Rev. Lett.* **67**, 1791–1794 (1991).
- Mason, T. E., Aeppli, G. & Mook, H. A. Magnetic dynamics of superconducting La_{1.86}Sr_{0.14}CuO₄. *Phys. Rev. Lett.* **68**, 1414–1417 (1992).
- Thurston, T. R. *et al.* Low-energy incommensurate spin excitations in superconducting La_{1.85}Sr_{0.15}CuO₄. *Phys. Rev. B* **46**, 9128–9131 (1992).
- Yamada, K. *et al.* Doping dependence of the spatially modulated dynamical spin correlations and the superconducting-transition temperature in La_{2–x}Sr_xCuO₄. *Phys. Rev. B* **57**, 6165–6172 (1998).
- Bulut, N., Hone, D., Scalapino, D. J. & Bickers, N. E. Random-phase approximation analysis of NMR and neutron-scattering experiments on layered cuprates. *Phys. Rev. Lett.* **64**, 2723–2726 (1990).
- Lu, J. P., Si, Q., Kim, J. H. & Levin, K. NMR relaxation and neutron scattering in a Fermi-liquid picture of the metallic copper oxides. *Phys. Rev. Lett.* **65**, 2466–2469 (1990).
- Littlewood, P. B., Zaanen, J., Aeppli, G. & Monien, H. Spin fluctuations in a two-dimensional marginal Fermi liquid. *Phys. Rev. B* **48**, 487–498 (1993).
- Tranquada, J. M., Sternlieb, B. J., Axe, J. D., Nakamura, Y. & Uchida, S. Evidence for stripe correlations of spins and holes in copper oxide superconductors. *Nature* **375**, 561–563 (1995).
- Tranquada, J. M. *et al.* Neutron-scattering study of stripe-phase order of holes and spins in La_{1.48}Nd_{0.4}Sr_{0.12}CuO₄. *Phys. Rev. B* **54**, 7489–7499 (1996).
- Hirota, K., Yamada, K., Tanaka, I. & Kojima, H. Quasi-elastic incommensurate peaks in La_{2–x}Sr_xCu_{1–y}Zn_yO_{4–δ}. *Physica B* **241/243**, 817–819 (1998).
- Lee, Y. S. *et al.* Neutron scattering study of spin density wave order in the superconducting state of excess-oxygen-doped La₂CuO_{4+y}. *Phys. Rev. B* (in the press).
- Zimmerman, M. V. *et al.* Hard X-ray diffraction study of charge stripe order in La_{1.48}Nd_{0.4}Sr_{0.12}CuO₄. *Europhys. Lett.* **41**, 629–634 (1998).
- Tranquada, J. M., Buttery, D. J. & Sachan, V. Incommensurate stripe order in La_{2–x}Sr_xNiO₄ with $x = 0.225$. *Phys. Rev. B* **54**, 12318–12323 (1996).
- Moncton, D. E., Axe, J. D. & DiSalvo, F. J. Neutron scattering study of the charge-density wave transitions in 2 H-TaSe₂ and 2 H-NbSe₂. *Phys. Rev. B* **16**, 801–819 (1977).
- Pintschovius, L. & Reichardt, W. in *Physical Properties of High-Temperature Superconductors IV* (ed. Ginsberg, D. M.) 295–372 (World Scientific, Singapore, 1993).
- Reichardt, W. Cu-O bond-stretching vibrations in YBa₂Cu₃O₇ studied by inelastic neutron scattering. *J. Low Temp. Phys.* **105**, 807–812 (1996).
- Reznik, D., Keimer, B., Doğan, F. & Asay, I. A. q -Dependence of self-energy effects of the plane oxygen vibration in YBa₂Cu₃O₇. *Phys. Rev. Lett.* **75**, 2396–2399 (1995).
- Pyka, N. *et al.* Superconductivity-induced phonon softening in YBa₂Cu₃O₇ observed by inelastic neutron scattering. *Phys. Rev. Lett.* **70**, 1457–1460 (1993).
- McQueeney, R. J. *et al.* Anomalous dispersion of LO phonons in La_{1.85}Sr_{0.15}CuO₄ at low temperatures. *Phys. Rev. Lett.* **82**, 628–631 (1999).
- Zachar, O., Kivelson, S. A. & Emery, V. J. Landau theory of stripe phases in cuprates and nickelates. *Phys. Rev. B* **57**, 1422–1426 (1998).
- Dai, P., Mook, H. A. & Doğan, F. Incommensurate magnetic fluctuations in YBa₂Cu₃O_{6.6}. *Phys. Rev. Lett.* **80**, 1738–1741 (1998).

Acknowledgements

We thank W.-T. Lee and J. L. Robertson for discussions on phonon dispersion in striped phases, and P. Dai for discussions on magnetic excitations. The work at Oak Ridge was supported by the US Department of Energy.

Correspondence and requests for materials should be addressed to H.A.M. (e-mail: ham@ornl.gov).

A ferromagnet having no net magnetic moment

H. Adachi^{††} & H. Ino^{‡‡}

^{*} Department of Materials Science, the University of Tokyo, Tokyo 113, Japan

[†] Institute of Materials Structure Science, KEK, Tsukuba, Ibaraki 305, Japan

[‡] College of Engineering, Hosei University, Koganei, Tokyo 184, Japan

Coupling between the spins (magnetic moments) of assemblies of ions can lead to ordered magnetic systems—classified as ferromagnetic, antiferromagnetic, ferrimagnetic and so forth, depending on the nature of the ordering¹. A simple picture of the coupling and ordering is usually adequate for describing properties of these systems, such as the magnitude and thermal variation of the magnetization. Here we describe a system whose magnetic behaviour appears counterintuitive on the basis of this picture. The materials in question are based on the ferromagnetic compound, SmAl_2 , in which some of the magnetic samarium ions, Sm^{3+} , have been substituted with other rare-earth elements. The resulting system exhibits the seemingly incompatible properties of large spin polarization but no bulk magnetization: this state occurs at a specific temperature, when the two components of the magnetization (the electron's spin and its orbital motion) exactly compensate for one another. This property should be generic to ferromagnets containing trivalent samarium ions, and may find potential application in, for example, spin-resolving devices for charged particles.

One of the rare-earth ions, Sm^{3+} , carries a unique magnetic moment. The spin and orbital magnetic moments, each having a considerable size of $\sim 4\mu_B$, are coupled antiparallel by the spin-orbit interaction and almost cancel out. Furthermore, the temperature dependences of the two are slightly different from each other. This feature is explained basically in terms of the J multiplet of the $4f$ electrons, which is the usual way to discuss rare-earth magnetism. For most of the rare-earth elements, where only the ground J multiplet is concerned with the magnetic properties, both the spin and orbital moments can be expressed by a single operator of the total angular momentum in the quantum mechanical description. This implies that they have the same temperature dependence. For Sm^{3+} , however, a few low multiplets having different J must be considered² because of the narrow energy intervals and the relatively large matrix elements of the spin and/or orbital angular momentum operators between the consecutive J multiplets. The spin and orbital moments then have to be treated as distinct operators; the admixture among nearby multiplets due to various interactions in solids, and the thermal population which extends over the excited multiplets, make a slight deviation between their temperature dependences. Note that the J -mixing itself also varies with temperature as causal action just as the exchange field does. The different temperature dependences of the spin and orbital moments which thus arise, combined with the delicate balance between them, can show up in the unique temperature dependence of the total magnetic moment. In the case of a ferromagnet, whose magnetization arises mainly from the Sm^{3+} ion, the resultant magnetization is, in general, quite small and its thermal variation has a marked resemblance to the characteristic variations of ferrimagnets^{3,4} (Fig. 1). At a specific temperature, perfect compensation between the spin and orbital parts of the magnetization could in theory be attained, where the rather large spin moments are entirely in a ferromagnetic order and the net magnetization is zero. These properties are of considerable interest from the viewpoint of the possible technological applications, as well as the fundamental natures of the material.

A ferromagnetic Sm compound with such a compensation

temperature, however, has not yet been found, although many compounds are known in which either the spin or the orbital magnetization barely exceeds the other throughout the ordered states^{3,4}. In order to establish guidelines for the control of the compensation temperature as a first step, we have studied the effects of substitution for Sm in the ferromagnetic cubic Laves-phase compound SmAl_2 , in which the orbital moment is slightly larger than the spin moment⁴. The substitution elements were selected to be non-magnetic Sc, Y, and La, and magnetic Nd and Gd; these elements have the same number of electrons to be delivered to the conduction band, which avoids a drastic change in magnetic properties, such as the collapse of the ferromagnetic order. Our original expectations were as follows. (1) The non-magnetic substituents were anticipated to expand or contract the lattice of the mother compound because of the difference in size, and the lattice variations might bring about the change of the conduction-electron component of the spin moment⁵. Such a change might cause spin-orbital compensation because the conduction-electron polarization has a significant contribution to the overall small magnetic moment of Sm^{3+} (refs 6–9); (2) it is empirically known, as a general rule, that substitution of the other magnetic rare-earth elements for a particular one in a magnetically ordered solid maintains (anti)ferromagnetic coupling among the spin moments^{10,11}. In the present case of SmAl_2 , then, since the spin moment contributes negatively to the total magnetization, the substitution of Nd (for which, too, the orbital moment dominates the spin one) was expected to increase the resultant magnetization, and an appropriate amount of Gd (for which the spin moment dominates or the orbital moment is absent), on the contrary, to decrease it, causing spin-orbital compensation.

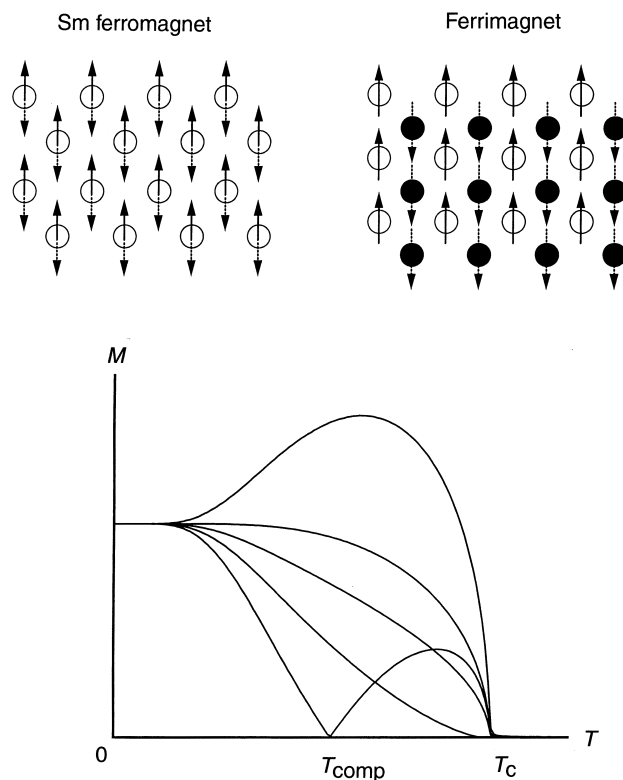


Figure 1 Diagrams of moment alignments. The various types of the magnetization (M) versus temperature (T) curves expected for a Sm ferromagnet and a ferrimagnet are shown. Solid and dotted arrows denote, respectively, the spin and orbital parts of the Sm^{3+} moment in the upper left figure. Those in the upper right figure indicate the magnetic moments of two kinds of ions (circles and filled circles). T_c is the ordering temperature, and T_{comp} is the compensation temperature.

Some magnetic properties are summarized in Fig. 2 as a function of the concentration of the substituents x . All the samples of single phase were prepared by argon-arc melting. With the substitution of non-magnetic elements, very little change in shape was observed in their magnetization versus temperature curves regardless of the expansion or the contraction of lattices. On the other hand, the effects of Nd and Gd were as we expected (Fig. 3). What is of importance is that these pseudo-ferrimagnetic curves originate mainly from the different temperature dependences of the spin and orbital magnetizations, and that the spin moments of the constituent rare-earths are coupled ferromagnetically throughout the ordered phase even at the compensation temperatures, which are observed to be 81 K for $(\text{Sm}_{0.982}\text{Gd}_{0.018})\text{Al}_2$ and 64 K for $(\text{Sm}_{0.974}\text{Gd}_{0.026})\text{Al}_2$. For spin-dominant ferromagnetic systems, such as SmZn and SmCd (refs 4, 8), adding Nd would probably cause a similar 'dip' in the magnetization versus temperature curves, due to the spin-orbital compensation.

One of the interesting practical applications of the present Sm ferromagnet may be spin-resolving devices for charged particles. Ferromagnetic materials in general seem suitable for such devices because of their uniform spin polarization of electrons. In many cases, however, the so-called magnetostatic effect prevents their practical use. Under no external magnetic field, ferromagnetic substances tend to split into small domains, each of which is magnetized in a different direction from the neighbouring ones, stabilized by lowering the overall magnetostatic energy. In the case of a soft-magnetic material, therefore, the uniform polarization of the electron spin is not established over a macroscopic range without the aid of an external field. However, the presence of a magnetic field concomitant with the formation of a single domain, whether an external field or a stray one from the surface of the material, acts on the charge or spin of a particle through the Lorentz and magnetic force, and could be a serious disturbance of the spin-resolving process. The present zero-magnetization ferromagnet is expected not to suffer from this effect. Its applicability to the analyser or polarizer of electron or positron spin, the probe tip of a spin-dependent scanning tunneling or exchange-force microscope, and so on, seems an interesting subject to be investigated. Another interesting idea may be the growth of multilayers having

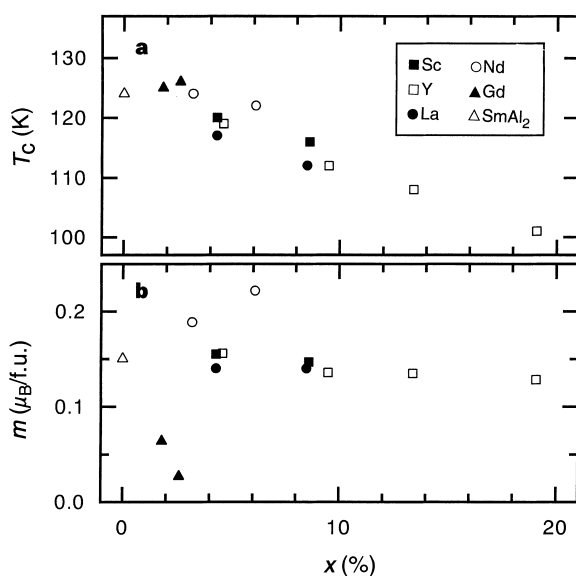


Figure 2 Magnetic properties plotted against substituent concentration. **a**, The ordering temperatures, and **b**, the spontaneous magnetizations at 2 K. Results are shown for pulverized samples of SmAl_2 (open triangles) and also pseudo-binary compounds $(\text{Sm}_{1-x}\text{R}_x)\text{Al}_2$ (indicated by their substituents, where R is Sc, Y, La, Nd or Gd). The measurements were made using a SQUID magnetometer (f.u., formula unit).

zero-moment ferromagnetic layers. Reduction of the magnetization, while keeping the interlayer exchange coupling, or the spin-analysing or polarizing power for an electric current, might enable us to observe what has been hidden by the electromagnetic interaction. It is obvious that the compensation state with an antiferromagnetic spin structure, as observed for many conventional ferrimagnets, is less suitable for these uses. Although the prospects are speculative at present, these materials might indicate a new field of technology and basic research, making use of the spin state of charged particles.

Thus far, we consider that the persistence of the ferromagnetic spin ordering at the magnetization 'dip' is unquestionable, given the ferromagnetic nature of the relevant system family, and that the stability of the spin direction is ensured by the strong adherence of the Sm^{3+} moment to a specific direction, a result of the electrostatic interaction between the surrounding ions and the anisotropic charge distribution of Sm^{3+} . This persistence and stability of the spin ordering implies that the experimental geometry, and the preceding control of temperature and an external field, can lead to the condition with arbitrary spin direction and no magnetization or stray fields. However, it is difficult to prove that the desired spin ordering is really there; yet essential, as the aforementioned ideas are dependent on this point. As far as we know, the best solution will be given by a magnetic Compton scattering experiment using synchrotron radiation X-rays⁹. In such an experiment, only the spin part of the magnetization would be detectable and the magnitude of the spin moment can be directly obtained. One difficulty is that, as the sample has no net magnetization, we cannot reverse the sample spin direction by an external magnetic field, in order to extract information about the spin. Instead, we would have to switch the degree of

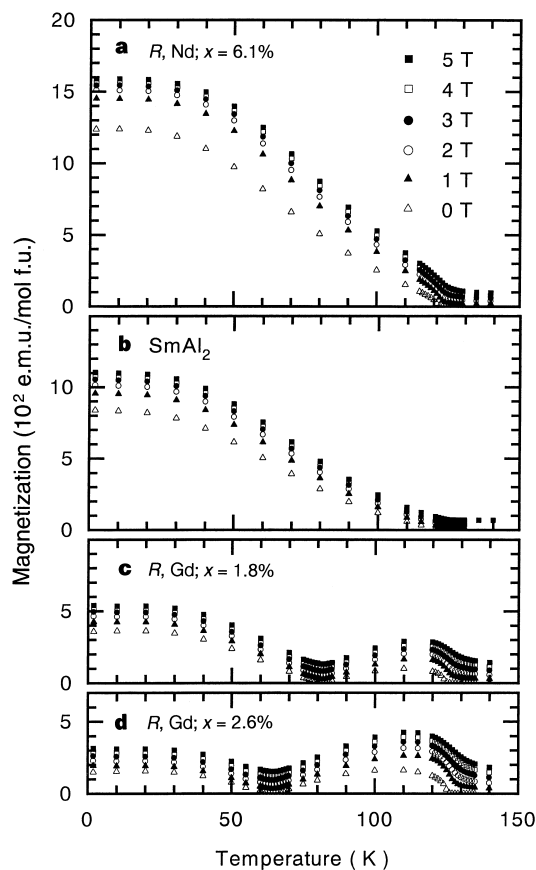


Figure 3 Magnetization versus temperature curves. Data was plotted for pulverized samples of SmAl_2 and pseudo-binary compounds $(\text{Sm}_{1-x}\text{R}_x)\text{Al}_2$ (where R is Nd or Gd). The data were taken from the low-temperature side, with the field changing from 5 T to zero at each temperature.

circular polarization of the incident X-rays, giving an equivalent effect. Even so, with the recent development of the synchrotron radiation technology, we should be able to perform such an experiment. □

Received 29 January; accepted 13 July 1999.

- Chikazumi, S. *Physics of Ferromagnetism*. (Oxford Univ. Press, 1997).
- Van Vleck, J. H. *The Theory of Electric and Magnetic Susceptibilities*, Sec. 59 (Oxford University Press, 1932).
- Adachi, H., Ino, H. & Miwa, H. Effect of conduction-electron polarization on the magnetism of hcp samarium metal. *Phys. Rev. B* **56**, 349–354 (1997).
- Adachi, H., Ino, H. & Miwa, H. Separation of the 4f-spin, 4f-orbital and conduction-electron magnetization from exotic thermomagnetic behavior of ferromagnetic Sm intermetallics. *Phys. Rev. B* **59**, 11445–11449 (1999).
- Legvold, S. *et al.* Magnetic properties of gadolinium-rich alloys. *Phys. Rev. B* **16**, 4986–4989 (1977).
- Stewart, A. M. The ordered moment of metallic samarium materials. *Phys. Status Solidi B* **52**, K1–K4 (1972).
- Koehler, W. C. & Moon, R. M. Magnetic structures of samarium. *Phys. Rev. Lett.* **29**, 1468–1472 (1972).
- Givord, D., Morin, P. & Schmitt, D. Unusual form factor in SmZn. *Phys. Lett. A* **73**, 221–223 (1979).
- Adachi, H. *et al.* Evidence for positive polarity of the spin moment in hcp Sm determined from a magnetic Compton-scattering experiment. *Phys. Rev. B* **56**, R5744–R5746 (1997).
- Williams, H. J., Wernick, J. H., Nesbitt, E. A. & Sherwood, R. C. Magnetic properties of rare earth aluminum compounds with MgCu₂ structure. *J. Phys. Soc. Jpn* **17** (suppl. B-1), 91–95 (1962).
- Grover, A. K., Malik, S. K., Vijayaraghavan, R. & Shimizu, K. Hyperfine fields in Sm_{1-x}Gd_xAl₂ alloys—Microscopic evidence for ferromagnetic coupling between rare earth spins. *J. Appl. Phys.* **50**, 7501–7503 (1979).

Acknowledgements

We thank H. Miwa, K. Kimura, S. Shin, A. Kakizaki and H. Kawata for discussions. T. Yatsu helped to prepare the samples. Magnetic measurements were made at the Cryogenic Center of the University of Tokyo. This work was partially supported by the Japan Society for Promotion of Science, a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan.

Correspondence and requests for materials should be addressed to H.A. (e-mail: hiromichi.adachi@kek.jp).

Unidirectional rotary motion in a molecular system

T. Ross Kelly, Harshani De Silva & Richard A. Silva

Department of Chemistry, E. F. Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, USA

The conversion of energy into controlled motion plays an important role in both man-made devices and biological systems. The principles of operation of conventional motors are well established, but the molecular processes used by 'biological motors' such as muscle fibres, flagella and cilia^{1–9} to convert chemical energy into co-ordinated movement remain poorly understood^{10–12}. Although 'brownian ratchets'^{13–16} are known to permit thermally activated motion in one direction only, the concept of channelling random thermal energy into controlled motion has not yet been extended to the molecular level. Here we describe a molecule that uses chemical energy to activate and bias a thermally induced isomerization reaction, and thereby achieve unidirectional intramolecular rotary motion. The motion consists of a 120° rotation around a single bond connecting a three-bladed subunit to the bulky remainder of the molecule, and unidirectional motion is achieved by reversibly introducing a tether between the two units to energetically favour one of the two possible rotation directions. Although our system does not achieve continuous and fast rotation, the design principles that we have used may prove relevant for a better understanding of biological and synthetic molecular motors producing unidirectional rotary motion.

A range of molecular devices^{17–23}, including turnstiles, shuttles and switches, has been synthesized, but a molecule able to produce unidirectional rotary motion has not yet been reported. Our attempts to design a molecular system able to achieve such motion focused on structure **1** (Fig. 1), which consists of two main components: a three-bladed triptycene (the lighter-shaded unit in Fig. 1b) and a [4]helicene (the darker-coloured component in Fig. 1b). The triptycene and helicene are connected by a single bond (shown as a black wedge in **1**; Fig. 1a) that functions as an axle. We have previously established^{24–27} that the barrier to rotation (~25 kcal mol⁻¹) of the triptycene around the triptycene/helicene bond in **1** is substantially higher than the barrier to rotation around typical carbon–carbon single bonds (~3–5 kcal mol⁻¹). The helicene in **1** may be regarded as a stiff (but not entirely rigid) pawl that resists deformation and prevents easy rotation. Molecule **1** cannot be looked upon as a molecular ratchet, however, because when thermally stimulated rotation of the triptycene does occur, it occurs in clockwise and anti-clockwise directions to an equal extent^{24,25}. The helicene in **1** might best be pictured as a friction brake²⁸ that inhibits, but does not completely prevent, spontaneous rotation of the triptycene.

The essence of achieving unidirectional rotation with a molecular system based on the structure of **1** involves continuing the friction-braking action to prevent anti-clockwise rotation, while using the chemical energy of carbonyl dichloride to lower the energy barrier of this system against clockwise rotation, thereby selectively fostering formally induced clockwise rotation of the triptycene. The unidirectional rotation derives ultimately from the asymmetric skew of the helicene and the use of a monosubstituted triptycene, which result in non-identical energy surfaces for clockwise and anti-clockwise rotation. To us, the function of the carbonyl dichloride is reminiscent of that of another energy-rich molecule, ATP, which powers many biological motors.

The thermodynamics of the design are presented in detail in Fig. 2. The basic strategy was to start with a molecule related to **1**, which has a rotational energy barrier of ~25 kcal mol⁻¹ (see Fig. 2b) but can be trapped (schematically represented by a 'brick wall' in Fig. 2d) somewhat above the rotational ground state by using a chemical reaction powered by carbonyl dichloride. The chemically trapped species is therefore energetically closer (compare Fig. 2d

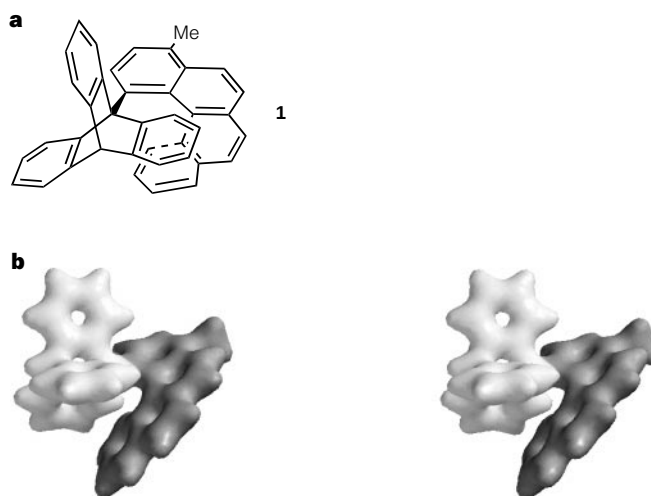


Figure 1 Compound **1**, a triptycyl[4]helicene. **a**, Chemical structure. **b**, Stereo view of a calculated electron-density surface map of the lowest-energy conformation of **1** using the Spartan (see below) version of the AM1 semi-empirical molecular modelling program. The triptycene is shown in lighter tones, the helicene in darker tones. The calculated barrier to rotation ($\Delta H^\ddagger = 22$ kcal mol⁻¹) around the triptycene/helicene bond in **1**—calculated using version 4.0 (1995) of the Spartan software (Wavefunction Inc., Irvine, California, USA)—is in good agreement with the experimentally determined value²⁵ ($\Delta G^\ddagger = 25$ kcal mol⁻¹).

with 2b) to the rotation barrier and requires relatively smaller amounts of thermal activation to reach the summit of the rotational energy barrier. Once the system has reached the summit (Fig. 2e), further rotation brings it into the second rotational ground state (Fig. 2f) and releases 25 kcal mol⁻¹. This energy immediately diffuses through the system, and thus is not directly available to drive the more energy-intensive reverse reaction (which requires 25 kcal mol⁻¹, in contrast to the less energy demanding forward process from the state shown in Fig. 2d to that in Fig. 2f). Put

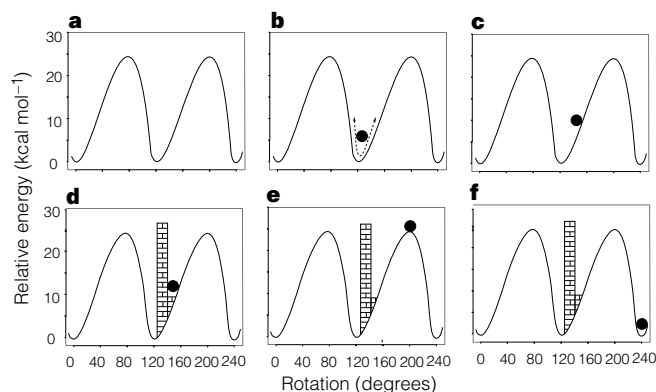


Figure 2 Schematic representation of the concepts underlying the design of the system. **a**, Energy diagram representing 240° of rotation around the triptycene/helicene bond (black wedge in **1**), with a barrier of ~25 kcal mol⁻¹. **b**, At any given time a single molecule (represented by the filled black circle) will usually exist in a low-energy conformation such as ~120° (≡~0° and ~240° because of the three-fold symmetry of the triptycene) around the triptycene/helicene bond. **c**, Within a very brief time span, random thermal energy temporarily elevates all individual molecules to conformationally excited states (for example, $t_{1/2}$ for thermally exciting molecules 10 kcal mol⁻¹ above ground state at 25 °C is 2.3×10^{-6} s). **d**, Conformationally excited rotamers in **c** are trapped, and prevented from rotating back to a lower-energy conformation. **e**, The trapped molecules are propelled by the random thermal energy to the top of the energy barrier (at energy E_{act}) (reversion to the position in **d**—but not **b**—is possible, but readily reversible). **f**, Descent from the summit in **e** to the next energy minimum is easy and virtually irreversible (because the reverse reaction (**f**→**e**) has an energy requirement of +25 kcal mol⁻¹, which is effectively inaccessible ($t_{1/2}$ for achieving +25 kcal mol⁻¹ is 63.2 h at 25 °C).

another way, the states shown in Fig. 2b and f are isoenergetic, and the rates of the b → f and f → b conversions will be identical; but by using the chemical energy of carbonyl dichloride to trap (Fig. 2d) the molecule as shown in Fig. 2c, the transformation c → f is exoergic, and unidirectional rotation is driven by the negative change in free energy for the c → f transformation.

Figure 3 provides molecular detail for the concepts outlined in Fig. 2. The demonstration of controlled rotary motion starts with **2** (the synthesis and characterization of all compounds will be reported elsewhere). Compound **2** is one of three low-energy rotational isomers (rotamers) about the axle connecting the triptycene and helicene components (**7** is a second low-energy rotamer). Rotamer **2** is activated by reaction with carbonyl dichloride to give the isocyanate **3** via a carbamoyl chloride. Isocyanate **3** is chemically prepared to react with the OH group in the hydroxypropyl tether attached to the helicene, but in the rotational ground state **3**, the isocyanate and the OH group are too far apart to interact. However, at those instants when a clockwise rotation of the triptycene (not possible with a comparable anti-clockwise rotation because an anti-clockwise rotation would bring the reactants further apart and prevent the essential urethane formation) brings the isocyanate and the OH group sufficiently close to react (see **4**), urethane formation (→**5**) can then result, irreversibly trapping the triptycene in a relatively high-energy (compared with **3**; also see Fig. 2d versus b) conformation around the triptycene/helicene axle. Ambient thermal energy then drives the exoergic unidirectional rotation from **5** to **6** (in contrast, but as expected, the control experiment involving

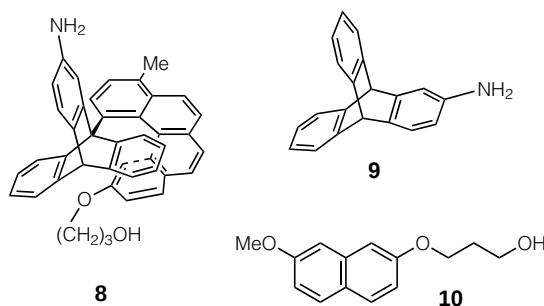


Figure 4 Molecules **8**, **9** and **10**, referred to in the text and in Fig. 5.

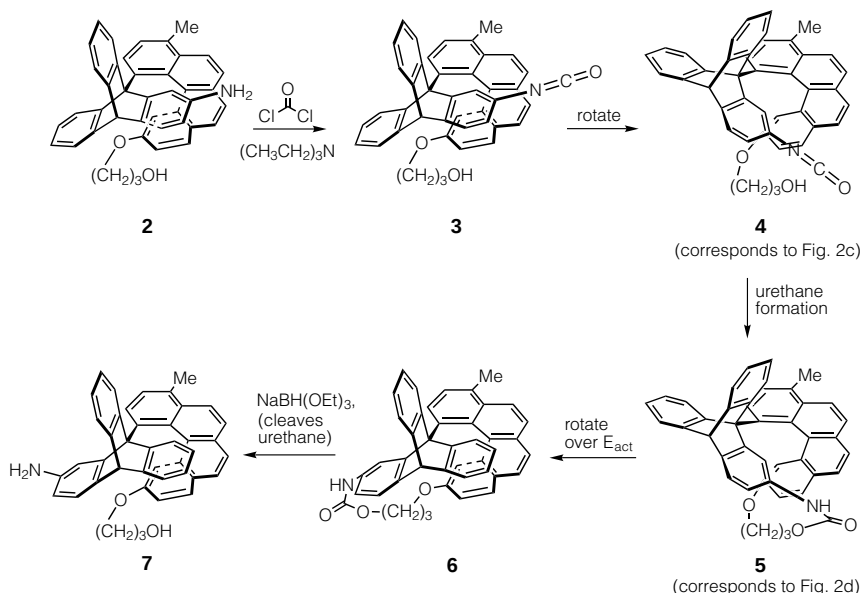


Figure 3 Sequence of events in the chemically powered rotation of **2** to **7**. See text for discussion. Although **2** and **3** are not identical, to a first approximation they both

conceptually correspond to Fig. 2b. Compounds **4** and **5** correspond to Fig. 2c and d, respectively; compounds **6** and **7** both correspond roughly to Fig. 2f.

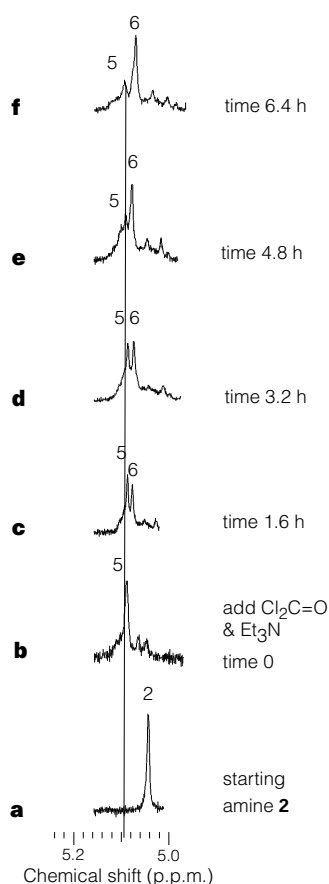


Figure 5 Spectroscopic evidence that carbonyl dichloride fuels the unidirectional rotation of **2**. Partial ^1H NMR spectra (monitoring the bridgehead proton relative to $(\text{CH}_3)_4\text{Si}=\text{O}$ p.p.m.) indicate the sequence of events as a function of time. Numbers next to peaks in the spectra refer to structures in Fig. 3. **a**, **2** in CDCl_3 ; **b**, t_0 ; addition of $\text{Cl}_2\text{C}=\text{O}$ and Et_3N . **2** is rapidly converted to intramolecular urethane **5** via isocyanate **3**; the isocyanates **3** and **4** convert to **5** too rapidly to be seen in the spectra. **c**, After 1.6 h at ambient temperature ($\sim 22^\circ\text{C}$), $\sim 30\%$ of **5** has rotated "over the hump" (Fig. 2d to Fig. 2f) to **6**. **d**, **e**, **f**, Over further time, rotation of **5** to **6** continues, with unidirectional conversion of **5** to **6** being $>80\%$ complete in ~ 6 h. Urethane **6** was isolated and shown to be identical to material prepared directly from amine rotamer **7** by reaction with $\text{Cl}_2\text{C}=\text{O}/\text{Et}_3\text{N}$. Control experiments established that **6** does not convert to **5**; that is, that the conversion of **5** $\rightarrow$ **6** is unidirectional. The assignment of structures to **3**–**6** is corroborated by infrared spectroscopic monitoring of the relevant reactions of **2**, **7** and **9** with $\text{Cl}_2\text{C}=\text{O}/\text{NEt}_3$ (and also **10** in the case of **9**), performed using ReactIR technology (ASI Applied Systems, Millersville, Maryland, USA); this equipment makes it possible to record *in situ*, and in real time, the infrared spectra of reaction mixtures and to thereby assay functional group changes as they occur.

treatment of **7** with $\text{Cl}_2\text{C}=\text{O}/\text{Et}_3\text{N}$ leads rapidly to **6**, but **6** does not detectably convert to **5**). Finally, **6** is cleaved to give **7**, thereby completing the chemically driven rotation of **2** to **7**. Control experiments demonstrate that in the absence of $\text{Cl}_2\text{C}=\text{O}/\text{Et}_3\text{N}$, pure **2** slowly converts (over several days) to an approximately 1:0.8:1 equilibrium mixture of **2**, **7** and **8** (the third low-energy rotamer: see Fig. 4). In Fig. 5 we show the experimental data which establish that the events in Fig. 3 proceed as indicated. $\square$

Received 11 March; accepted 16 July 1999.

1. Rayment, I. *et al.* Structure of the actin-myosin complex and its implications for muscle contraction. *Science* **261**, 58–65 (1993).
2. Abrahams, J. P., Leslie, A. G. W., Lutter, R. & Walker, J. E. Structure at $2.8 \text{ \AA}$ resolution of $\text{F}_1\text{-ATPase}$ from bovine heart mitochondria. *Nature* **370**, 621–628 (1994).
3. Dominguez, R., Freyzon, Y., Trybus, K. M. & Cohen, C. Crystal structure of a vertebrate smooth muscle myosin motor domain and its complex with the essential light chain: visualization of the pre-power stroke state. *Cell* **94**, 559–571 (1998).
4. Block, S. M. Real engines of creation. *Nature* **386**, 217–219 (1997).
5. Noji, H., Yasuda, R., Yoshida, M. & Kinoshita, K. Jr Direct observation of the rotation of $\text{F}_1\text{-ATPase}$.

Nature **386**, 299–302 (1997).

6. Boyer, P. D. The ATP synthase—a splendid molecular machine. *Annu. Rev. Biochem.* **66**, 717–749 (1997).
7. Shingyoji, C., Higuchi, H., Yoshimura, M., Katayama, E. & Yanagida, T. Dynein arms are oscillating force generators. *Nature* **393**, 711–714 (1998).
8. Berg, H. C. Keeping up with $\text{F}_1\text{-ATPase}$. *Nature* **394**, 324–325 (1998).
9. Vale, R. D. & Oosawa, F. Protein motors and Maxwell's demons: does mechanochemical transduction involve a thermal ratchet? *Adv. Biophys.* **26**, 97–134 (1990).
10. Stryer, L. in *Biochemistry* Ch. 15, 4th edn (W. H. Freeman, New York, 1995).
11. Howard, J. Molecular motors: structural adaptations to cellular functions. *Nature* **389**, 561–567 (1997).
12. Huxley, A. How molecular motors work in muscle. *Nature* **391**, 239–240 (1998).
13. Faucheux, L. P., Bourdieu, L. S., Kaplan, P. D. & Libchaber, A. J. Optical thermal ratchet. *Phys. Rev. Lett.* **74**, 1504–1507 (1995).
14. Travis, J. Making light work of Brownian motion. *Science* **267**, 1593–1594 (1995).
15. Astumian, R. D. Thermodynamics and kinetics of Brownian motion. *Science* **276**, 917–922 (1997).
16. Rousselet, J., Salome, L., Ajdari, A. & Prost, J. Directed motion of brownian particles induced by a periodic asymmetric potential. *Nature* **370**, 446–448 (1994).
17. Mislou, K. Molecular machinery in organic chemistry. *Chemtracts—Org. Chem.* **2**, 151–174 (1989).
18. Bedard, T. C. & Moore, J. S. Design and synthesis of molecular turnstiles. *J. Am. Chem. Soc.* **117**, 10662–10671 (1995).
19. Balzani, V., Gomez-Lopez, M. & Stoddart, J. F. Molecular machines. *Acc. Chem. Res.* **31**, 405–414 (1998).
20. Sauvage, J.-P. Transition metal-containing rotaxanes and catenanes in motion: toward molecular machines and motors. *Acc. Chem. Res.* **31**, 611–619 (1998).
21. Bissell, R. A., Cordova, E., Kaifer, A. & Stoddart, J. F. A chemically and electrochemically switchable molecular shuttle. *Nature* **369**, 133–137 (1994).
22. Benniston, A. C. & Harriman, A. A light-induced molecular shuttle based on a [2]rotaxane-derived triad. *Angew. Chem. Int. Edn. Engl.* **32**, 1459–1461 (1993).
23. Mao, C., Sun, W., Shen, Z. & Seeman, N. C. A nanomechanical device based on the B-Z transition of DNA. *Nature* **397**, 144–146 (1999).
24. Kelly, T. R., Tellitu, I. & Sestelo, J. P. In search of molecular ratchets. *Angew. Chem. Int. Edn. Engl.* **36**, 1866–1868 (1997).
25. Kelly, T. R., Sestelo, J. P. & Tellitu, I. New molecular devices: in search of a molecular ratchet. *J. Org. Chem.* **63**, 3655–3665 (1998).
26. Davis, A. P. Tilting at windmills? The second law survives. *Angew. Chem. Int. Edn. Engl.* **37**, 909–910 (1998).
27. Musser, G. Taming Maxwell's demon. *Sci. Am.* **280**, 24 (1999).
28. Kelly, T. R. *et al.* A molecular brake. *J. Am. Chem. Soc.* **116**, 3657–3658 (1994).

Acknowledgements

We thank S. Jasmin and Y. Zhao for contributions to the preparation of necessary quantities of **2**, and J. Sieglén and B. Wang for technical assistance. This work was supported by the NIH.

Correspondence and requests for materials should be addressed to T.R.K. (e-mail: ross.kelly@bc.edu).

Light-driven monodirectional molecular rotor

Nagatoshi Koumura^{*†}, Robert W. J. Zijlstra^{*}, Richard A. van Delden^{*}, Nobuyuki Harada[†] & Ben L. Feringa^{*}

^{*} Department of Organic and Molecular Inorganic Chemistry, Stratingh Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

[†] Institute for Chemical Reaction Science, Tohoku University, 2-1-1 Katahira, Aoba, Sendai 980-8577, Japan

Attempts to fabricate mechanical devices on the molecular level^{1,2} have yielded analogues of rotors³, gears⁴, switches⁵, shuttles^{6,7}, turnstiles⁸ and ratchets⁹. Molecular motors, however, have not yet been made, even though they are common in biological systems¹⁰. Rotary motion as such has been induced in interlocked systems^{11–13} and directly visualized for single molecules¹⁴, but the controlled conversion of energy into unidirectional rotary motion has remained difficult to achieve. Here we report repetitive, monodirectional rotation around a central carbon–carbon double bond in a chiral, helical alkene, with each 360° rotation involving four discrete isomerization steps activated by ultraviolet light or a change in the temperature of the system. We find that

axial chirality and the presence of two chiral centres are essential for the observed monodirectional behaviour of the molecular motor. Two light-induced *cis-trans* isomerizations are each associated with a 180° rotation around the carbon-carbon double bond and are each followed by thermally controlled helicity inversions, which effectively block reverse rotation and thus ensure that the four individual steps add up to one full rotation in one direction only. As the energy barriers of the helicity inversion steps can be adjusted by structural modifications, chiral alkenes based on our system may find use as basic components for 'molecular machinery' driven by light.

The structure of the molecular rotor (3*R*,3'*R*)-(P,P)-*trans*-1,1',2,2',3,3',4,4'-octahydro-3,3'-dimethyl-4,4'-biphenanthrylidene (**1** in Fig. 1) features two identical halves connected by a central carbon-carbon double bond. (Here *P* denotes the right-handed helicity in each half of the structure; *M*, used later, stands for left-handed helicity.) The design of the molecular rotor presented here is based on the exploitation of two fundamental principles. First, the light-induced *trans* to *cis* isomerization around a carbon-carbon double bond is an extremely fast and reversible process; in nature, it forms the basis for the information-retrieval step in the process of vision. Second, the concerted action of two chiral elements in a single chemical or physical event can lead to a unique handedness via the process of chiral discrimination, one of the essential features in living organisms. Steric interference in the molecule imposes a helical shape to the structure. *Trans*-1 and *cis*-2 are isomeric structures following a 180° rotation around the central bond, a process which is accomplished by irradiation with ultraviolet light at room temperature.

The configuration at the stereogenic centre, bearing the methyl-substituent, is (*R*) for both halves; Me_{ax} and Me_{eq} (Fig. 1) are axial and equatorial oriented methyl-substituents, respectively. The synthesis of *trans*-(3*R*,3'*R*)-(P,P)-**1** and the unequivocal determination of the absolute stereochemistry and molecular structures of *trans*-1 and

cis-2 have been reported¹⁵. We studied the photochemical switching behaviour of **1**, and Fig. 1 shows the dynamic processes and the different stereoisomeric structures that were observed. The absorption spectra of the stereoisomers of **1** and **2** are given in Fig. 2.

Irradiation (wavelength $\lambda \geq 280$ nm) of (P,P)-*trans*-1 in *n*-hexane at -55 °C resulted in the formation of (M,M)-*cis*-2. A *cis*-2 to *trans*-1 ratio of 95:5 is observed at photoequilibrium. The isomerization is fully reversible, and the photoequilibrium shifts to (P,P)-*trans*-1 as the main isomer on irradiation with $\lambda \geq 380$ nm. The *trans*-*cis* photoisomerization is evident from ¹H NMR analysis, and the simultaneous reversal of *P* to *M* helicity is detected by circular dichroism (CD) (Fig. 3, traces A and B).

When the temperature of the solution of (M,M)-*cis*-2 is raised to 20 °C, a fast and selective conversion to (P,P)-*cis*-2 is observed and the concomitant change in CD absorption (Fig. 3, traces B and C) shows the helix reversal associated with this thermal interconversion. Irradiation of (P,P)-*trans*-1 under ambient conditions leads to complete conversion to (P,P)-*cis*-2, as the unstable (M,M)-*cis*-2 is removed from the photoequilibrium in a fast thermal isomerization. Although (P,P)-*cis*-2 was prepared by photochemical isomerization of (P,P)-*trans*-1 (ref. 16) we have now established that there is no direct pathway between these isomers. It should be emphasized that the (M,M)-*cis*-2 to (P,P)-*cis*-2 isomerization is irreversible under the thermal or photochemical conditions that were employed. Subsequent irradiation of (P,P)-*cis*-2 at $\lambda \geq 280$ nm results in the formation of (M,M)-*trans*-1, reaching a photoequilibrium with a ratio of (M,M)-*trans*-1 to (P,P)-*cis*-2 of 90:10. The change in CD absorption that accompanies this photochemical *cis-trans* isomerization (Fig. 3, traces C and D) is again associated with a *P,P* to *M,M* helicity reversal. When the temperature of a solution of (M,M)-*trans*-1 was increased to 60 °C, (P,P)-*trans*-1 was formed exclusively and the CD absorptions again change sign, reverting to the original values found in the spectrum of (P,P)-*trans*-1. The four changes in the sign of the CD absorption occur only around 217 nm (Fig. 3). The reverse mode of this process (that is, direct photochemical or thermal isomerization of (P,P)-*trans*-1 to (M,M)-*trans*-1) is not observed.

The main question is why the thermal *M,M* to *P,P* double helix reversal for both the *trans*-isomer **1** and the *cis*-isomer **2** are irreversible. Molecular modelling studies (MOPAC 93-AM1, Fujitsu, Tokyo, 1993) of (3*R*,3'*R*)-*trans*-1 show two stable forms, (P,P)-*trans*-1 and (M,M)-*trans*-1, with an energy difference (ΔE) of 8.6 kcal mol⁻¹ in favour of the (P,P)-isomer in accordance with the experimental observations. In the more stable (P,P)-isomer, the two methyl substituents are in axial positions (to prevent steric hindrance) whereas in the less stable (M,M)-isomer the methyl groups adopt equatorial orientations. For the *cis*-isomer **2** the two methyl groups are again in an axial orientation in the most stable (P,P)-*cis*-2 form,

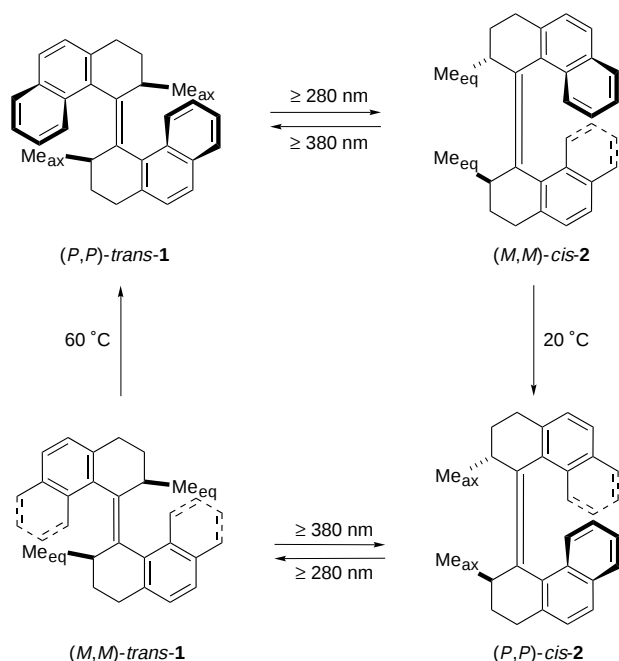


Figure 1 Photochemical and thermal isomerization processes of (P,P)-*trans*-1. UV irradiation with high pressure Hg-lamp, Pyrex filter, $\lambda \geq 280$ nm or Xe-lamp, Toshiba L-3g glass filter, $\lambda \geq 380$ nm. First order kinetics were observed for the thermal processes and temperature dependent ¹H NMR and CD studies in the range 50.0–81.1 °C gave $E_a = 26.4$ kcal mol⁻¹ for the (M,M)-*trans*-1 to (P,P)-*trans*-1 interconversion. It should be noted that no racemization takes place during any of the photochemical or thermal steps as was proven by chiral HPLC analysis of the isomers obtained after the individual steps.

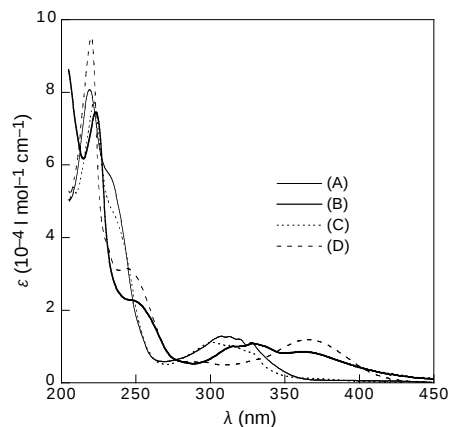


Figure 2 Ultraviolet-visible spectra (hexane solvent). Trace A, (P,P)-*trans*-1; trace B, (M,M)-*cis*-2; trace C, (P,P)-*cis*-2; trace D, (M,M)-*trans*-1.

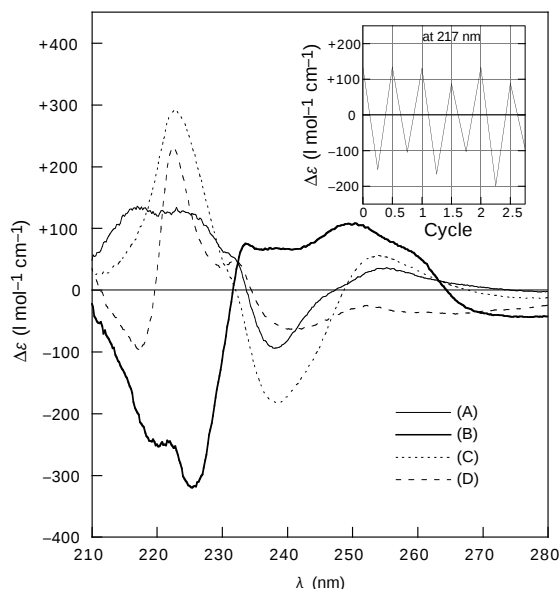


Figure 3 Circular dichroism (CD) spectra system in each of four stages of switching (see text). Trace A, (*P,P*)-*trans*-**1**; trace B (*M,M*)-*cis*-**2**; trace C, (*P,P*)-*cis*-**2**; trace D, (*M,M*)-*trans*-**1**. Inset, change in CD signal during full rotation cycle of (*P,P*)-*trans*-**1** monitored at 217 nm.

whereas in the less stable (*M,M*)-*cis*-**2** form ($\Delta E = 11.0 \text{ kcal mol}^{-1}$) the two methyl groups take equatorial positions.

The above results indicate that irradiation of (*P,P*)-*trans*-**1** at $\lambda \geq 280 \text{ nm}$ at 60°C will result in the formation of all three other states of the four-step isomerization cycle (shown in Fig. 1) consecutively, because under these conditions both photoisomerization steps as well as the two thermal isomerization steps will take place. The inset of Fig. 3 shows the change in CD signal at 217 nm, as monitored during three full cycles. When one considers the structural changes observed during the full cycle shown in Fig. 1 it is evident that, relative to the bottom half of the molecule (considered the static part), the top half of the molecule (considered the rotor) undergoes a full 360° rotation exclusively in a clockwise sense (as seen from the side of the static part). To the best of our knowledge, this is the first observation of a monodirectional photochemically driven rotation of a propeller-type unit, and it shows that controllable rotatory motion can be produced by the influx of external energy¹⁷.

The stereochemical changes during the entire rotation cycle that dictate the clockwise monodirectional behaviour for (*P,P*)-*trans*-**1** can be rationalized as follows. Photochemical *trans*–*cis* isomerization inverts the *P,P* helicity to *M,M* helicity. Simultaneously, the axial methyl substituents in (*P,P*)-*trans*-**1** adopt the less favourable equatorial orientation in (*M,M*)-*cis*-**2**. The photochemically induced—energetically uphill—process is followed by a thermal downhill process in which inversion of *M,M* to *P,P* helicity occurs, and the methyl substituents adopt the more favourable axial orientation. Next, a second photochemical step converts *cis*-**2** to *trans*-**1** with simultaneous *P,P* to *M,M* helix inversion; but in this step again, as a consequence of the rotation around the alkene double bond, the methyl groups adopt the less favourable equatorial orientation. Finally, (*M,M*)-*trans*-**1** relaxes to the starting isomer (*P,P*)-*trans*-**1** with simultaneous *M,M* to *P,P* helix inversion and a change of the less favourable equatorial to the more favourable axial orientation of the methyl groups.

The absorption of light energy and the unique combination of axial chirality and two chiral centres in this molecular rotor are essential for the observed monodirectional behaviour. Two light-driven steps induce rotations around the central alkene double bond and generate the less stable (*M,M*)-*cis*-**2** and (*M,M*)-*trans*-**1** isomers. Subsequently two energetically downhill processes generate the more stable (*P,P*)-*cis*-**2** and (*P,P*)-*trans*-**1** isomers. As the helicity inverts simultaneously in the last two steps, the reverse rotation pathway is effectively

blocked. Starting with (*3R,3'R*)-(*P,P*)-**1**, clockwise rotation is therefore found; if the (*3S,3'S*)-(*M,M*)-**1** enantiomer of this molecular rotor were used, anti-clockwise rotation is expected.

The cycle shown in Fig. 1 may be regarded as four distinct states in an optical molecular switch, each of which can be populated depending upon temperature and wavelength of the light used; at the appropriate wavelength and temperature, a continuous monodirectional rotation is induced in the system. The origin of this rotation process lies in the molecular architecture of structure **1**, where the following stereochemical features are all important: thermally stable, but photochemically interconvertible *cis* and *trans* isomers; (*P,P*) or (*M,M*) helicity; the fixed (*3R,3'R*)-configuration at the stereogenic centres; conformational flexibility of the cycloalkene rings; and the axial or equatorial orientation that can be adopted by the methyl groups. □

Received 3 June; accepted 2 August 1999.

1. Feynman, R. P. in *Miniaturization* (ed. Gilbert, H. D.) (Reinhold, New York, 1961).
2. Drexler, K. E. *Nanosystems: Molecular Machinery, Manufacturing and Computation* (Wiley, New York, 1992).
3. Schoevaars, A. M. *et al.* Toward a switchable molecular rotor. *J. Org. Chem.* **62**, 4943–4948 (1997).
4. Clayden, J. & Pink, J. H. Concerted rotation in a tertiary aromatic amide: towards a simple molecular gear. *Angew. Chem. Int. Edn Engl.* **37**, 1937–1939 (1998).
5. Huck, N. P. M., Jager, W. F., de Lange, B. & Feringa, B. L. Dynamic control and amplification of molecular chirality by circularly polarized light. *Science* **273**, 1686–1688 (1996).
6. Bissell, S. A., Córdova, E., Kaifer, A. E. & Stoddart, J. F. A chemically and electrochemically switchable molecular shuttle. *Nature* **369**, 133–136 (1994).
7. Ashton, P. R. *et al.* Acid-base controllable molecular shuttles. *J. Am. Chem. Soc.* **120**, 11932–11942 (1998).
8. Bedard, T. C. & Moore, J. S. Design and synthesis of a “molecular turnstile”. *J. Am. Chem. Soc.* **117**, 10662–10671 (1995).
9. Kelly, T. R., Tellitu, I. & Sestelo, J. P. In search of molecular ratchets. *Angew. Chem. Int. Edn Engl.* **36**, 1866–1868 (1997).
10. Noji, H., Yasuda, R., Yoshida, M. & Kinosita, K. Jr Directed observation of the rotation of F1-ATPase. *Nature* **386**, 299–302 (1997).
11. Balzani, V., Gómez-López, M. & Stoddart, J. F. Molecular machines. *Acc. Chem. Res.* **31**, 405–414 (1998).
12. Sauvage, J.-P. Transition metal-containing rotaxanes and catenanes in motion: toward molecular machines and motors. *Acc. Chem. Res.* **31**, 611–619 (1998).
13. Armaroli, N. *et al.* Rotaxanes incorporating two different coordinating units in their thread: synthesis and electrochemically and photochemically induced molecular motions. *J. Am. Chem. Soc.* **121**, 4397–4408 (1999).
14. Gimzewski, J. K. *et al.* Rotation of a single molecule within a supramolecular bearing. *Science* **281**, 531–533 (1998).
15. Harada, N., Koumura, N. & Feringa, B. L. Chemistry of unique chiral olefins. 3. Synthesis and absolute stereochemistry of *trans*- and *cis*-1,1',2,2',3,3',4,4'-octahydro-3,3'-dimethyl-4,4'-biphenanthrylidene. *J. Am. Chem. Soc.* **119**, 7256–7264 (1997).
16. Koumura, N. & Harada, N. Photochemistry and absolute stereochemistry of unique chiral olefins, *trans*- and *cis*-1,1',2,2',3,3',4,4'-octahydro-3,3'-dimethyl-4,4'-biphenanthrylidene. *Chem. Lett.*

1151–1152 (1998).

17. Davis, A. P. Tilting at windmills? The second law survives. *Angew. Chem. Int. Edn Engl.* 37, 909–910 (1998).

Correspondence and requests for materials should be addressed to B.L.F. (e-mail: B.L.Feringa@chem. rug.nl).

Acknowledgements

Financial support from the Netherlands Organisation for Scientific Research (NWO-STW) to B.L.F. is acknowledged.

Peacocks lek with relatives even in the absence of social and environmental cues

Marion Petrie^{*}, Andrew Krupa[†] & Terry Burke[†]

^{*} Evolution and Behaviour Research Group, Department of Psychology, University of Newcastle, Newcastle-upon-Tyne NE1 7RU, UK

[†] Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK

Lek mating systems are characterized by males displaying in groups. The main benefit from group display is thought to be an increase in the number of females arriving per male. However, when mating success is highly skewed it is not clear why unsuccessful males participate in group display¹. In theory, all males on leks could obtain indirect fitness benefits if displaying groups consisted of related individuals². Here we present two independent sets of data that show that peacocks (*Pavo cristatus*) display close to their kin. DNA fingerprinting showed that males at Whipsnade Park were more closely related to males within the same lek than to males at other leks. Separately, we found that after an experimental release of a mixed group of related and unrelated males, brothers (paternal sibs or half-sibs) established permanent display sites very close together. This result is unex-

pected, as the released birds could not become familiar with their brothers during their development. The released young were hatched from eggs that had been removed from their parents shortly after laying and mixed with the eggs of non-relatives. These data indicate that birds can evolve a means of kin association that does not involve learning the characteristics of relatives or the use of environmental cues. If social learning is not necessary for kin association then kin effects may be of more widespread importance in avian social interactions, and in particular in the evolution of lek mating, than previously appreciated.

Males in lek mating systems aggregate to display to attract females. Group display has been shown to increase the number of females arriving per male in several lekking species^{3,4}, but, as a result of the skew in mating success, this does not always increase the number of matings for all of the participants under all circumstances⁵. Unsuccessful males appear only to be increasing the mating success of their more successful neighbours. However, they may gain inclusive fitness benefits from their display if they lek in association with relatives, and lekking may therefore be promoted by kin selection². Here, we investigate whether peacock leks consist of relatives.

Peacocks were studied at Whipsnade Park, UK, where there is a population of around 200 free-ranging peafowl. Peacocks are a classic lekking species where groups of males aggregate at display sites and call together. Once females arrive on leks, males stop calling and display their upper tail coverts⁶; mating success is highly skewed, and most males on leks gain no matings. Peacocks establish permanent display sites in their fourth year. Males are present on their display sites for most of every day for the duration of the mating season, and return to the same site every year. Males can be as close as 2.5 m apart and, on one lek site in the park containing 10 individuals, males were on average 8.83 m (s.d. 6.50 m) apart⁶. We took blood samples from 21 displaying males distributed across four main lek sites (Fig. 1). We used multilocus fingerprinting to compare the genetic similarity (measured as the degree of band-sharing) within and between these lek sites. The degree of band-sharing within leks was significantly higher than that between leks (within leks mean $S = 0.816$, $n = 48$; between leks $S = 0.777$, $n = 162$; Mantel randomization test⁷, 100,000 randomizations, $P = 0.01$; Fig. 2). Assuming that birds in different leks are unrelated and the detected minisatellites segregate independently, the increased band sharing within leks is close to that expected for half-siblings (0.810)⁸.

How could peacocks come to display near to their close kin? Peacocks take no part in reproduction after mating and therefore young birds cannot learn the identity of their fathers. One possibility that could result in a tendency for related birds to display



Figure 1 Distribution of 59 displaying male peacocks at Whipsnade Park in 1995 (excluding released males). Four leks (green, red, purple and yellow) were defined in the study area according to close visual contact between displaying males. Adjacent birds excluded from leks (blue) were obscured by topography, stands of trees or fencing.

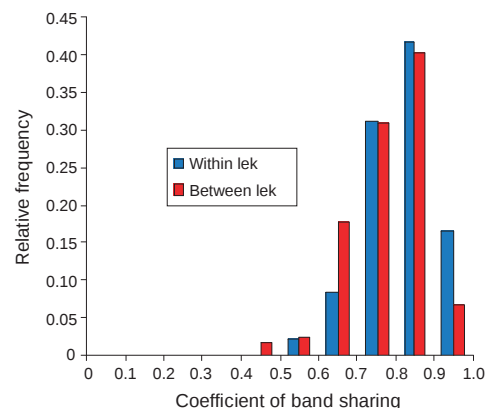


Figure 2 Comparison of band-sharing between individual multilocus minisatellite DNA profiles within (blue bars) and between (red bars) the display sites represented in Fig. 1. Peacocks displaying at the same lek ($n = 4, 6, 4$ and 7 , respectively) were significantly more genetically similar to one another than they were to birds at other leks ($P = 0.01$).

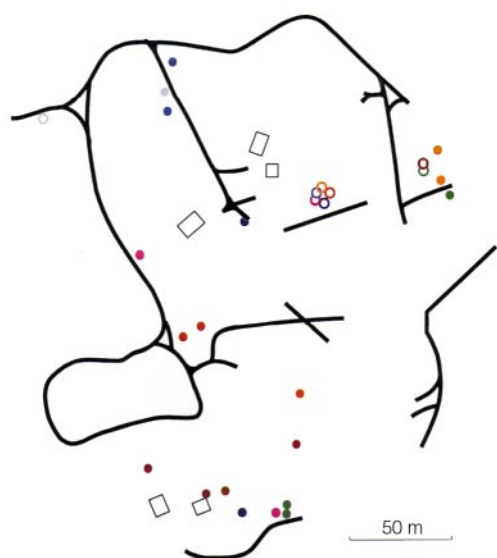


Figure 3 Permanent display sites established by all 19 peacocks of known paternity four years after their release into the study area (filled circles). Peacocks of the same paternity, and the ex-display site of each of their eight fathers (open circle) are represented in the same colour. On average, released birds of the same paternity established display sites that were significantly closer than those of different paternity ($P = 0.01$; Fig. 4), and became nearest neighbours far more often than expected by chance ($P = 0.0002$).

together is that there might be limited dispersal by males from their natal sites. We considered this possibility by testing whether it was necessary for peacocks to have been born in the park in order for them to display close to their relatives. We did this by plotting the display positions of a sample of young of mixed relatedness that were released into the park after being reared elsewhere. Our data allowed us to reject this possibility.

Figure 3 shows the positions of the display sites of 19 four-year-old males in 1995 (filled circles) that were released into Whipsnade Park as part of an experiment designed to look at the survivorship of the offspring of sires of differing attractiveness⁹. In 1991, eight full-trained displaying males of varying attractiveness were removed from Whipsnade Park (the open circles in Fig. 3 show the positions of the ex-display sites of these sires). These males were each allowed to breed with four females at a farm in Norfolk, UK. To control for rearing differences, eggs were removed from their parents, marked and incubated in groups of mixed relatedness. Individually marked young were kept in large mixed groups until they were old enough for a sample to be released into Whipsnade Park, early in 1992. The young of known paternity were released in batches of eight, each consisting of one offspring from each of the eight sires. After release, the young flocked and ranged together in large groups. Unexpectedly, given that the offspring could not be familiar with their relatives, when the birds established their permanent adult display sites several years after their release there was a clear tendency for known brothers or half-brothers to display close together (Fig. 3). Using a Mantel matrix randomization test, we found that the pairwise distances between relatives (paternal sibs/half-sibs) were significantly shorter than those between non-relatives (mean distance between pairs of paternal sibs/half sibs = 117 m, s.d. = 97 m, $n = 16$; mean distance between non-relatives = 183 m, s.d. = 99 m, $n = 137$; 100,000 randomizations, $P = 0.01$; Fig. 4). Although relatives were on average significantly closer than expected, the frequency of relatives that were nearest neighbours was particularly high. Considering just the display sites of the 19 birds released in the experiment, eight were closest to a paternal relative, when only 1.48 was expected ($P = 0.001$; Fig. 3). When the pre-existing displaying males (Fig. 1) were also included in the analysis, six of the released birds had paternal relatives as nearest

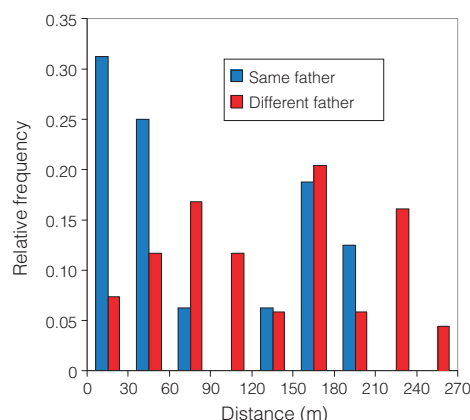


Figure 4 Comparison of distances between the permanent display sites of related (blue bars) and unrelated (red bars) experimentally released males (see Fig. 3; excludes the one male without a brother).

neighbours, when only 0.39 was expected ($P = 0.0002$).

It is possible that birds would be more familiar with the young that hatched in the same batch on the same day and this could influence their tendency to associate. However, there was no tendency for birds that were briefly reared together in the same batch to establish display sites close together (mean = 2.57 males per batch; Mantel test, 100,000 randomizations, $P = 0.59$).

One possible explanation for these data could be that the released brothers (full or half) tended to display close together because related individuals have the same genetic preference for particular environmental features. However, this seems unlikely on this small scale and in this homogeneous, non-native environment; moreover, it might then be expected that the offspring would also share such a genetic preference with their fathers. There is no obvious tendency for the released males to establish display sites near to their fathers' ex-display sites (birds are significantly closer to their nearest brother than to their father's ex-display site, $t = 2.44$, $P < 0.029$, $n = 18$).

These two independent sets of data show that peacocks display close to their kin. There is also evidence that male kin associate at leks in the black grouse, *Tetrao tetrix*, where it is suggested that this arises through limited natal dispersal by males¹⁰. In peafowl, our experimental release data show that lekking males will display next to their relatives even in the absence of any learnt social or environmental cues to their identity. They could use self-referent phenotype matching¹¹, where peacocks match heritable cues in their own phenotypes with those in other individuals. Evidence for kin discrimination in the absence of social learning has rarely been obtained¹²⁻¹⁸ and, to our knowledge, these data provide the first such evidence for birds^{18,19}. Although social learning is not apparently necessary for kin association, this does not necessarily mean that it is never used when available.

The results indicate that there could be advantages to displaying close to kin. It may be that the inclusive fitness benefits of cooperating with relatives to attract mates outweigh any costs of communal display. It is also possible that females prefer to choose mates from among a group of related individuals, and that the evolution of group displays by relatives is female-driven. Evidence for a purely genetic basis to kin association indicates that kin selection and inbreeding avoidance could be more important in the evolution of avian mating systems and other aspects of behaviour than has been previously appreciated. □

Methods

Multilocus DNA fingerprints were prepared from blood samples as described²⁰. Genomic DNA samples were digested with *MboI* (applied Biotechnologies), run on 1% agarose gels, blotted onto Magna Charge (Micon Separations) nylon membranes and probed with Jeffreys' minisatellite probe 33.6 using a stringency of $1 \times \text{SSC}/0.1\% \text{ SDS}$ at 65°C . There

was a high degree of band-sharing between individuals, probably due to past inbreeding in this population. The large number of monomorphic and common bands provided a reference ladder that allowed 28 apparently homologous polymorphic minisatellite fragments in the size range 4.0–23.0 kilobases (kb) to be identified between fingerprints, thus enabling the ready comparison of all individuals. The order of the samples was randomized within and between gels, and the band patterns were scored by an assistant who had no knowledge of the lek sites of the individual birds.

Eight free-ranging full-trained displaying lek males whose mating success varied were removed from Whipsnade Park during February 1991 and transferred to a peacock farm in Norfolk, UK. The peacocks were housed in separate pens and four naive adult peahens, known to be at least 2 years old, provided by the farm, were measured and randomly assigned to each pen on 14 March. Pens were checked daily for eggs (it was not possible to know which of the four hens laid which egg unless egg laying was observed) and any eggs found were labelled and removed. Groups of eggs originating from several different pens over several dates were mixed and placed under broody domestic chickens for incubation. Eggs were removed from the hens after 26 days and placed in a hatcher in batches, where each egg had its own compartment; each of the hatched chicks was given an individual colour ring combination. Each batch of eggs was incubated and hatched separately at approximately weekly intervals from May to August. Each batch of chicks was provided with a heat lamp and food and water *ad libitum*; batches were subsequently pooled and reared together. Females produced 519 eggs and the growth of the surviving 349 offspring was monitored. In January and February 1992, 12 offspring (7 males and 5 females) from each of the 8 males (3 from each of the 4 females per male) were introduced into Whipsnade Park. A matched sample of young was chosen from each pen so that there were no overall significant differences in hatching dates or weights of the offspring between fathers (at day 84, $F_{7,95} = 0.838$, $P = 0.559$; at introduction, $F_{7,95} = 0.358$, $P = 0.924$). Care was taken to release the offspring in batches of eight, consisting of one young of the same sex from each pen. The fate of the offspring was recorded by a field assistant who had no knowledge of the relatedness of any of the individuals, and the birds were observed every spring until they established permanent display sites in 1995 (aged 4). Of the introduced males, 19 were observed to have established permanent display sites in 1995 (Fig. 3).

Mantel tests⁷ that randomized the pairwise physical distances or band-sharing values, respectively, were performed on square-root transformed distances using the program RT v2.1 (ref. 21). We analysed nearest-neighbour associations using a program that randomized (100,000 times) the positions of relatives and non-relatives, as appropriate, and counted the number of occasions on which nearest neighbours were relatives.

Received 11 March; accepted 2 July 1999.

- Höglund, J. & Alatalo, R. V. *Leks* (Princeton Univ. Press, Princeton, 1995).
- Kokko, H., Lindström, J. Kin selection and the evolution of leks: whose success do young males maximise? *Proc. R. Soc. Lond. B* **263**, 919–923 (1996).
- Kokko, H., Sutherland, W. J., Lindström, J., Reynolds, J. D. & Mackenzie, A. Individual mating success, lek stability, and the neglected limitations of statistical power. *Anim. Behav.* **56**, 755–762 (1998).
- Alatalo, R. V., Höglund, J., Lundberg, A. & Sutherland, W. J. Evolution of black grouse leks—female preferences benefit males in larger leks. *Behav. Ecol.* **3**, 53–59 (1992).
- Widemo, F. & Owens, I. P. F. Lek size, male mating skew and the evolution of lekking. *Nature* **373**, 148–151 (1995).
- Petrie, M., Halliday, T. & Sanders, C. Peahens prefer peacocks with elaborate trains. *Anim. Behav.* **41**, 323–332 (1991).
- Manly, B. F. *Randomisation, Bootstrap and Monte Carlo Methods in Biology* 2nd edn (Chapman & Hall, London, 1997).
- Bruford, M. W., Hanotte, O., Brookfield, J. F. Y. & Burke, T. in *Molecular Genetic Analysis of Populations: A Practical Approach* 2nd edn (ed. Hoelzel, A. R.) 287–336 (IRL, Oxford, 1998).
- Petrie, M. Improved growth and survival of offspring of peacocks with more elaborate trains. *Nature* **371**, 598–599 (1994).
- Höglund, J., Alatalo, R. V., Lundberg, A., Rintamäki, P. T. & Lindell, J. Microsatellite markers reveal the potential for kin selection on black grouse leks. *Proc. R. Soc. Lond. B* **266**, 813–816 (1999).
- Lacy, R. C. & Sherman, P. W. Kin recognition by phenotype matching. *Am. Nat.* **121**, 489–512 (1983).
- Grosberg, R. K. & Quinn, J. F. The genetic control and consequences of kin recognition by the larvae of a colonial marine invertebrate. *Nature* **322**, 457–459 (1986).
- Potts, W. K., Manning, C. J. & Wakeland, E. K. Mating patterns in semi-natural populations of mice influenced by MHC genotype. *Nature* **352**, 619–621 (1991).
- Getz, W. M. & Smith, K. B. Genetic kin recognition: honey bees discriminate between full and half sisters. *Nature* **302**, 148 (1983).
- Grafen, A. Do animals really recognise kin? *Anim. Behav.* **39**, 42–54 (1990).
- Alexander, R. D. Epigenetic rules and Darwinian algorithms: the adaptive study of learning and development. *Ethol. Sociobiol.* **11**, 241–303 (1990).
- Sherman, P. W. Multiple mating and kin recognition by self-inspection. *Ethol. Sociobiol.* **12**, 377–386 (1991).
- Sherman, P. W., Reeve, H. K. & Pfennig, D. W. in *Behavioural Ecology: An Evolutionary Approach*, 4th edn (eds Krebs, J. R. & Davies, N. B.) 69–97 (Blackwell Science, Oxford, 1997).
- Blaustein, A. R., Bekoff, M. & Daniels, T. J. in *Kin Recognition in Animals* (eds Fletcher, D. J. C. & Michener, C. D.) 287–332 (Wiley, New York, 1987).
- Hanotte, O., Burke, T., Armour, J. A. L. & Jeffreys, A. J. Hypervariable minisatellite DNA sequences in the Indian peafowl *Pavo cristatus*. *Genomics* **9**, 587–597 (1991).
- Manly, B. F. J. *RT, A Program for Randomization Testing, version 2.1*. (Centre for Applied Statistics and Mathematics, University of Otago, 1997).

Acknowledgements

This study was initiated while M.P. was at the Department of Zoology, University of Oxford and A.K. and T.B. were at the Department of Biology, University of Leicester. We thank the Zoological Society of London for permission to study the peafowl at Whipsnade

Wild Animal Park; L. Lovett for help at Whipsnade; Q. Spratt for allowing M.P. To work at his peacock farm; A. Williams for help at the farm; P. Carpenter for help in scoring gels; E. Bell and A. Askew for writing computer programs; J. Brookfield for advice; J. Futter for help with producing Figures; B. Hatchwell, M. Young, G. Roberts, F. Ratnieks and P. Watt for comments on the manuscript; and the NERC and the BBSRC for financial support.

Correspondence and requests for materials should be addressed to M.P. (e-mail: marion.petrie@ncl.ac.uk).

An epigenetic mutation responsible for natural variation in floral symmetry

Pilar Cubas*, Coral Vincent & Enrico Coen

John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

Although there have been many molecular studies of morphological mutants generated in the laboratory, it is unclear how these are related to mutants in natural populations, where the constraints of natural selection and breeding structure are quite different. Here we characterize a naturally occurring mutant of *Linaria vulgaris*, originally described more than 250 years ago by Linnaeus^{1–3}, in which the fundamental symmetry of the flower is changed from bilateral to radial. We show that the mutant carries a defect in *Lcyc*, a homologue of the *cycloidea* gene which controls dorsoventral asymmetry in *Antirrhinum*⁴. The *Lcyc* gene is extensively methylated and transcriptionally silent in the mutant. This modification is heritable and co-segregates with the mutant phenotype. Occasionally the mutant reverts phenotypically during somatic development, correlating with demethylation of *Lcyc* and restoration of gene expression. It is surprising that the first natural morphological mutant to be characterized should trace to methylation, given the rarity of this mutational mechanism in the laboratory. This indicates that epigenetic mutations may play a more significant role in evolution than has hitherto been suspected.

Mature wild-type flowers of *Linaria vulgaris* (toadflax) have five petals that are united for part of their length to form a corolla tube ending in five separate lobes (Fig. 1c, d). Dorsoventral asymmetry is clearly evident in the shape and colour of the petals. The two dorsal (adaxial) petals have relatively long strap-shaped lobes; the two lateral petals have wider lobes with a partially orange lip; and the ventral (abaxial) petal has a small lobe with an orange lip, and a spur-shaped nectary at its base. Dorsoventral asymmetry is also evident in the stamens: the dorsal stamen is arrested early in development to give a sterile staminode (Fig. 1d), and the two lateral stamens are shorter and less hairy than the two ventral stamens.

Flowers of naturally occurring peloric mutants in *Linaria* are radially symmetrical (Fig. 1a–d). All five petals resemble the ventral petal of wild type, each having a small lobe with an orange lip, and a spur at their base. Similarly, there are five stamens, all of which closely resemble the ventral stamens of wild type in length and hairiness. In being fully ventralized, these mutant *Linaria* flowers resemble peloric mutants of *Antirrhinum* which lack the activity of two related genes, *cycloidea* (*cyc*) and *dichotoma*^{4,5}. The peloric mutation in *Linaria* is recessive, as crosses to wild type yielded essentially wild-type F₁ progeny. Only one of the F₁ individuals occasionally gave one or two extra spurs.

We compared the development of wild-type and peloric flowers of *Linaria* by scanning electron microscopy. No differences were

* Present address: Centro Nacional de Biotecnología Campus de la Universidad Autónoma de Madrid, Cantoblanco, 28049, Madrid, Spain.

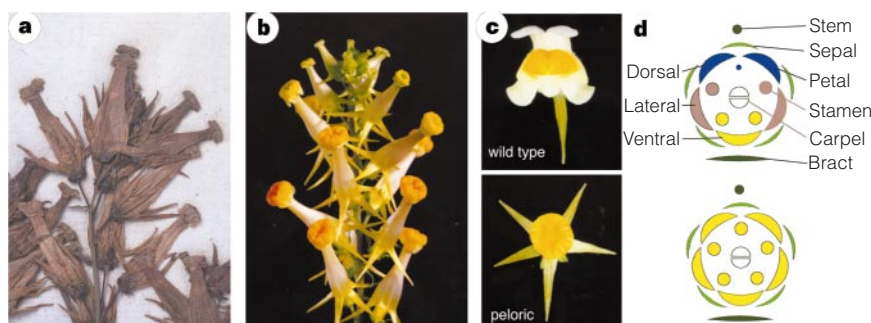


Figure 1 Wild-type and peloric *Linaria vulgaris* flowers. **a**, Original herbarium specimen of peloric *Linaria* inflorescence collected by Linnaeus and currently housed in the Linnean Society, London. **b**, Peloric *Linaria* inflorescence from a living specimen. **c**, Face view of a wild-type *Linaria* flower compared to a peloric mutant. **d**, Floral diagrams of wild-type (top) and peloric (bottom) flowers showing the relative positions of different organs, with

identities indicated by colours: blue (dorsal) brown (lateral) yellow (ventral). The wild-type flower has an axis of dorsoventral asymmetry orientated such that the dorsal (upper or adaxial) part is nearer the stem whereas the ventral (lower or abaxial) part is nearer to the subtending leaf. The peloric flower is radially symmetrical, with all petals resembling the ventral petal of the wild type.

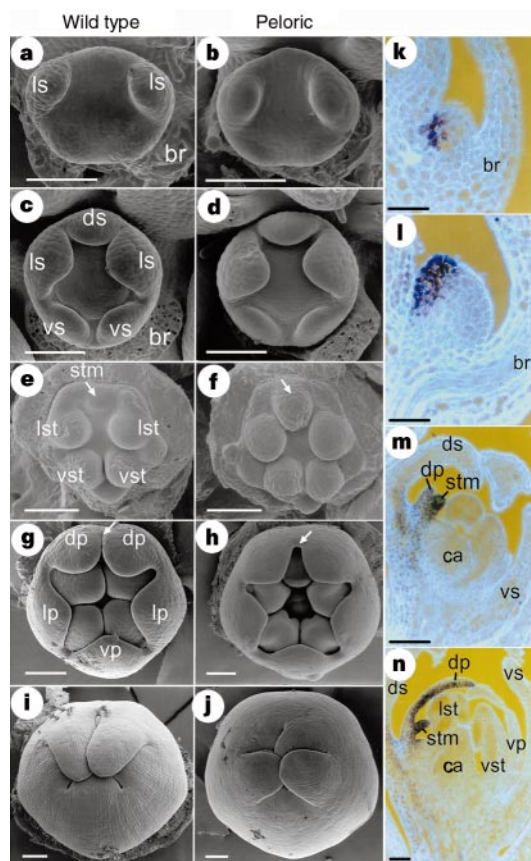


Figure 2 Development of wild-type and peloric flowers as revealed by scanning electron microscopy (SEM) and expression of *Lcyc* during floral development. **a–j**, Five stages studied by SEM are shown. No difference between mutant and wild type are observed at early stage 4 (**a, b**; sepal primordia initiated) or late stage 4 (**c, d**; all five sepal primordia are clearly visible). By early stage 6 (**e, f**; sepal primordia have been removed to show stamen primordia), the dorsal stamen (**stm**) of wild type was relatively small and retarded in growth (**e**); whereas in the peloric mutant, the dorsal stamen was indistinguishable from the other stamen primordia (**f**, arrow). Differences in petal development became evident at a slightly later stage, when the corolla tube and lobes started to form (**g, h**; stage 7). In wild type the dorsal petal lobes had a distinctive shape and were separated by a relatively narrow gap (**g**, arrow), whereas in the

peloric mutant, all the petal lobes were identical (**h**). At later stages, when petals enclose the stamens and carpel (**i, j**), the wild-type dorsal petal lobes consistently cover the other petals, whereas the petals of the mutant are more equivalent. **k–n**, RNA *in situ* hybridizations of wild-type floral meristems probed with *Lcyc*. All panels show longitudinal sections; the inflorescence stem is to the left. The signal is seen as dark blue. **k**, Stage 2; **l**, stage 3; **m, n**, later stages when all organ primordia are visible. Scale bars, 100 μ m. Stages are defined according to ref. 23. br, Bract primordium; ca, carpel primordium; dp, dorsal petal primordium; ds, dorsal sepal primordium; lp, lateral petal primordium; ls, lateral sepal primordium; lst, lateral stamen primordium; stm, staminode primordium; vp, ventral petal primordium; vs, ventral sepal primordium; vst, ventral stamen primordium.

seen early on, when sepal primordia were being initiated on the periphery of the floral meristem (Fig. 2a–d). However, differences were evident shortly after this, when stamen and petal primordia had emerged. The dorsal stamen primordium was retarded in wild type but not in peloric mutants (Fig. 2e, f). Similarly, the dorsal petal primordia had a distinctive shape in wild type but were similar

to the other petals in peloric mutants (Fig. 2g–j). These effects on early stamen and petal development are comparable to those seen for peloric mutants of *Antirrhinum*, although in the case of *Antirrhinum* there are also earlier effects on organ number⁴.

To investigate whether the peloric mutation might be caused by an alteration in a homologue of the *cyc* gene of *Antirrhinum*, a genomic

1 GATAAAGCTTGGGCCCCCTCTACACACACCTCCACACACTGGTCTAATTTGAAGTAAA 60
61 GACAACTAATATATGAATGACTGTAAGAAAAACGATGATTAACCCCTAGTGGTTTGGAC 120
122 CTGTATTGGATTAAACACATTCGATTCGATCCTGATGTAATGATGATGATACCCGCG 180
181 TGTGCTCTATTTTGTATGCTAAATATGTTTCTTATGATCGTCAGGGGTTGATAGGGC 240
241 CCCCCCTCTACTCCATCAGATGGTCTTCTTCTTACTACTTCTTATGATGATGATGAT 300
301 TAAATAAATCTCTTTTATTTCAATGGACATTAAGAGTACAACCAATGAATCATATATAT 360
361 TTGTGCTGAAGTGGACGAACCTGTTTGTAGGTACCAATGATGATGATGATGATGATGAT 420
421 GTTTTGTAAATAGTGTAGTACCAATTAATTAATATATACCATATATCTGAGCCAGA 480
481 TTTTGAAGCTTGTGGAGTTCTAAACCTTTTGAAGCAGAGAAAGTACAAAAATACTTTA 540
541 GTTATATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 600
601 TTGATATTCATTCATCCTCTCTTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 660
661 CACAGGATTAGTCTCTTTCATGTAACAAAGAAATGACAGCTTTATTTCTTCTCTGTC 720
721 CTCTCAATATAAATAAATCATGTTAATAGATGATATATTAATTCACCTCCTAAGAAAGG 780
781 TAAACAAAGAGCCCTATTTTCCATGGCAAGATCATAAACTCTTCAAGGATTTCTTCTCT 840
841 GAAACAGCTTCTATGAGCTCAAAATATAAGGGTTTCTTGTACCTCTCTTCAATCAT 900
901 CACTCCCAACAAAGGAAATAGAAAATGTTTGGGAAGACACATACCTACACCTT 960
301 M F G K N T Y L H L 320
961 CCTCATCAGGTTTCAACCTCTCTTAACTCTGTCGCGTGTGTGACCTAAACGGCAACGAG 1020
321 P H Q V S T S L N S R A V V D L N G N E 340
1021 ATCCAACTCCATCTCCAGCATCACCACACGACATCTCTCCGCTCACTACTTGACCAAC 1080
341 I Q L H L Q H H H D I L S G H Y L T N 360
1081 GGTCTGTTCTTGTAGTCCACAGCTTGTTCACCACTTCAACAATCAACATGTTGTAGT 1140
361 G P V L E S T A L F N F N N Q H V V S 380
1141 CACGGGATGAAGTCCATGATCTGCTGCAATATGCGAGAAATCTTCCAAACCAAA 1200
381 H G M N C H D P D P A N M A E T F Q T K 400
1201 CAAAGTACTGTGAAGAGGACCGGCACAGCAAGATATACACTGCTCAAGGCGGAGAGAC 1260
401 Q S T V K K D R H S K I Y T A Q G P R D 420
1261 AGGAGAGTTGCTGCTGCTCCATCGCATTCGAGGAGTCTTTGATCTACAAGAGATGCTA 1320
421 R R V R L S I G I A R K F F D L Q E M L 440
1321 GGTTCGACAGCAAGCAAGACCTCGACTGGCTCTTCTAAGTCGAAACACGACATA 1380
441 G P D K P S K T L D W L L T K S K T A I 460
1381 AAAGAGCTAGTGCAGTCCAAAGTACGAGAGCAATCTTCTAATCCCATCTCTGATGAT 1440
461 K E L V Q S K S T K S N S S N S H S D D 480
1441 GATTGTGATGATGAGGTTGTGCTGCGGAGGAGATTCTGAGAAGCTGCAAGATTGCAAG 1500
481 D C D D E V V S A E G D S E N A A D S K 500
1501 GGGAGTCCGCTGCTTATAAAGGTAAGTCAAAATGTAAGAAGCTGCTCTGCAATGGAT 1560
501 G K S V L I K G N Y K C K E A A S A M D 520
1561 TCTCAACAGCAGCACTCAATCTCGTGAAGAGTTCGAGGGCAAGGCGAGAGCAAGAGCT 1620
521 S Q Q A A L N L V K E S R A K A R A A 540
1621 AGGGAAGAACCAAGGAGAAATGTGTATCAACAACTGAATGAAGCTAGGAACAAAC 1680
541 R E R T K E K M C I K Q Q L N E A R N N 560
1681 AACAAGGTTGCTGACTGATCAATATCCCTTCAACATGTAATCCAGTCTAAGCAAGAAC 1740
561 N K G G D W I N N P F N N V I Q S N R N 580
1741 CATCAACAGTTCGAAAGTCGCGAGGCGGCTTTGTTCAATCCGCTTTTGGGTTTCAT 1800
581 H Q Q F E S R E A A F V H H P V F G F H 600
1801 CAGCAAACTACAGCAAGCATCTGCTCTCTCATGAGAACTGAGATCAGTCAAGCAG 1860
601 Q Q N Y S N A S A S S H E N W D Q S N Q 620
1861 CTATGCGCATTTTGAATCAGCAAGTTCATCAATAGGTAAAAGAAGAAATTTAAA 1920
621 L C A I L N Q H K F I N * 640
1921 CTATTTTATAAGTGTGTTTCCAAATTTTGGCCATACCAATGTCAGGTCAAATGTGATTTA 1980
1981 TGGTTTGTCTTTTGTCTAACAGCAAGTTGAACATAGGAACCTTTTGCATCTCTCAAGA 2040
2041 TCTCAAGGTCAGAAAGGTTCTTTTGTGAGAAATTTATGTTTGTGTACCTCTCTTT 2100
2101 TCCAGAGGTCATGAATCTTATCAATCTAATCTTATGATTTAAAAAGGTTATGAATA 2160
2161 GTATATTAGTATTGATCTAATTTATATCCATGTTTCTGCTAGATATATCAATTAACAA 2220
2221 CTCTGCTGATTATCTAATAATCTGCACTCAAGCTAATCATGTGACTGATTTTATGAGT 2280
2281 CTCTGATATGCTGTTTCCAAATATATGCAAGGTTATTTGCTTATGTGACCACTAAGC 2340
2341 ATGGAATTTTGTGTTTCAATCGAGGTATGAAAGCAGAGACAGCAAGCTACTATCATGTT 2400
2401 TAACATCAAAATCATGAGATAAGCTTG 2428

Figure 3 Sequence of *Lycy*. Wild-type genomic sequence containing the *Lycy* ORF. The similarity between *Lycy* and *cycloidea* is 87% identity at the DNA level. The deduced protein sequence is indicated underneath in the standard one-letter notation. Restriction sites that are methylated in peloric plants are underlined. *Hae*III GGCC; *Msp*I CCGG; *Pst*I, CTGAG; *Sau*3A1, GATC; *Ma*II, ACGT; *Dde*I, CTNAG (not all the *Dde*I sites were analysed). Site of nucleotide polymorphism in the 3' region is boxed.

clone of a *Linaria cyc*-like gene, *Lycy*, was isolated (Fig. 3) and used to probe DNA blots of an F₂ population segregating for peloria. Digestion with several restriction enzymes revealed restriction-fragment length polymorphisms (RFLPs) linked to the peloric phenotype, suggesting that the mutation was either within, or closely linked to, the *Lycy* gene (Fig. 4a, b). In *Antirrhinum*, a second gene, related to *cyc*, also needs to be inactivated to give a fully peloric phenotype^{4,5}, indicating that in *Linaria* there may be less redundancy with respect to the role of these genes in the control of floral asymmetry.

The effect of the peloric mutation on *Lycy* expression was determined by RNA *in situ* hybridization. In wild type, *Lycy* was expressed in the dorsal region of floral meristems from very early stages of development (Fig. 2k, l). At later stages, expression became restricted to the dorsal staminode and dorsal petal primordia (Fig. 2m, n). The overall expression pattern of *Lycy* was therefore similar to that of *cyc* in *Antirrhinum*⁴, consistent with a role for *Lycy* in establishing the asymmetry of the *Linaria* flower. No expression of *Lycy* was detected in floral meristems from peloric individuals (data not shown). Taken together with our RFLP analysis, this suggested that a mutation blocking *Lycy* expression was probably responsible for the peloric phenotype.

To determine the nature of this mutation, we compared the genomic sequences of *Lycy* in mutant and wild type. Apart from a

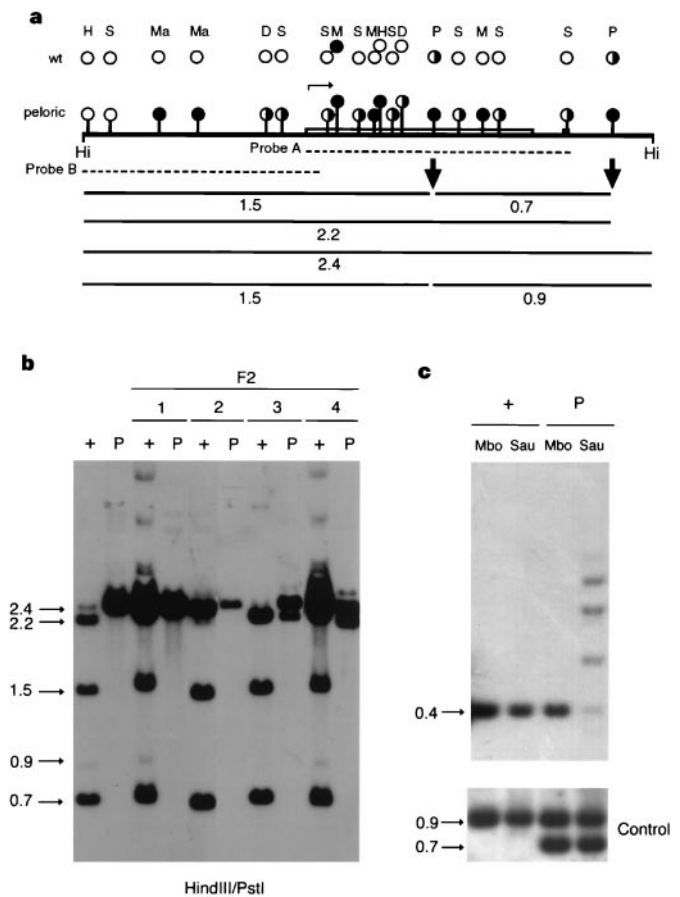


Figure 4 RFLP analysis and methylation of *Lycy*. **a**, Restriction map of the *Lycy* genomic region showing location of sites that are methylated in peloric plants. Lollipops indicate complete (filled), partial (half-filled) or no methylation (empty) at the sites. The ORF region is indicated by an open box. Probe A was used in **b** and **c** and in Fig. 5b; probe B was used to analyse the methylation at the promoter region of *Lycy*. Fragments resulting from complete digestion with *Hind*III and partial or complete digestion with *Pst*I due to methylation are shown. H, *Hae*III; Hi, *Hind*III; M, *Msp*I; S, *Sau*3A1; Ma, *Ma*II; D, *Dde*I. Numbers indicate the size of each fragment in kilobases. **b**, Genomic DNA from young leaves of wild-type (+) and peloric (P) individuals digested with *Hind*III/*Pst*I, blotted and hybridized with the *Lycy* probe A (see **a**). The first two lanes show DNA from the parents; other lanes show DNA from siblings with a wild-type or mutant phenotype taken from four different F₂ families (1–4). Based on a DNA-sequence polymorphism at the 3' end of *Lycy*, the wild-type F₂ segregants shown were heterozygous for the peloric allele. **c**, Genomic DNA of wild-type (+) and peloric (P) plants digested with *Mbo*I or *Sau*3A1, blotted and hybridized with probe A. In addition to the 0.4-kb fragment, three bands of 139 bp, 144 bp and 148 bp were expected but were too small to be resolved on the gel. Lower panel, same blot after stripping and hybridizing with a homologue of the *centroradialis* gene²⁴ from *Linaria* ('control': no other bands were visible on the blot). The 0.7-kb band hybridizing to *Lcentroradialis* in peloric plants corresponds to an RFLP present in this line. Similar blots were stripped and hybridized with a second *Lycy*-like gene and with a ubiquitin cDNA probe, which revealed no differences between wild-type and peloric restriction patterns (not shown).

nucleotide polymorphism in the 3' region (Fig. 3, boxed nucleotide), no sequence polymorphisms specific to the peloric mutant were found within the *Lycy* coding region or in the 930 base pairs (bp) of upstream sequence. Surprisingly, we observed no difference at restriction sites previously shown to be polymorphic by RFLP analysis (Fig. 3). Because the enzymes that detected RFLPs were sensitive to cytosine methylation, one explanation was that the mutant allele was methylated. We checked this by digesting wild-type and peloric genomic DNA with a pair of isoschizomers (*Mbo*I and *Sau*3A1) that differed in sensitivity to methylation. Probing with *Lycy* revealed no difference in digests with the insensitive enzyme

(*Mbo*I), whereas the methylation-sensitive enzyme *Sau*3A gave incomplete digestion of *Lcyc* specifically in the peloric mutant (Fig. 4c). This differential methylation did not extend to all genes, as similar blots probed with other *Linaria* genes revealed no difference between wild-type and mutant DNA (Fig. 4c). Thus, there is an association between plants carrying heavily methylated *Lcyc* and the peloric phenotype. However, even DNA from wild type showed evidence of some methylation (see bands at 2.2 and 2.4 kilobases (kb) in Figs 4b, 5b), perhaps reflecting somatic modification of *Lcyc* in a proportion of wild-type cells.

These results might be explained in two ways: either a methylated *Lcyc* allele from the peloric parent was being transmitted through the germ line to the F_2 , or *Lcyc* was being methylated *de novo* in a proportion of the F_2 progeny. These possibilities could be distinguished by following the segregation of a DNA-sequence polymorphism between the parents in the 3' region of *Lcyc*. Sequencing of this region in several peloric and wild-type F_2 segregants showed that all peloric individuals analysed were homozygous for the *Lcyc* allele from the peloric parent, whereas the wild types were either homozygous or heterozygous for the allele from the wild-type parent. Thus, the phenotypes in the F_2 appeared to reflect transmission of a modified allele of *Lcyc*.

Although these results indicated that the peloric mutation was linked to a methylated *Lcyc* allele, they did not prove that methylation affected *Lcyc* activity. For example, the methylated allele might have carried a DNA sequence change in an upstream region that was primarily responsible for reduced *Lcyc* expression. This could be tested by analysing peloric plants that showed somatic instability. Several F_2 peloric plants produced some branches with almost wild-type flowers, or flowers that were intermediate between peloric and wild type (semipeloric; Fig. 5a). Cuttings were taken from branches with different flower phenotypes, and the methylation of *Lcyc* was analysed in the propagated plants (Fig. 5b). The phenotype correlated with the state of *Lcyc* methylation: the nearly wild-type plants were partially demethylated, whereas the peloric and semipeloric plants were heavily methylated. Probing blots of DNA digested with methylation-insensitive 6-bp cutters (for example, *Hinc*II, *Xba*I) revealed no other alterations, such as excision of a transposon, around 10 kb of the *Lcyc* locus, although excisions of less than 200 bp could not be ruled out. RNA *in situ* hybridization showed that demethylation of *Lcyc* correlated with recovery of a wild-type *Lcyc* transcription pattern in all three layers of floral meristems (data not shown). Thus, the lack of *Lcyc* expression in the peloric plants was not due to a defect in the *Lcyc* DNA sequence, but to a heritable epimutation involving DNA methylation. This epimutation was not completely stable during somatic develop-

ment, occasionally reverting through demethylation.

It is surprising that the first natural morphological mutant to be characterized should trace to an epimutation because most mutations recovered from studies of laboratory stocks are due to DNA-sequence alterations or transpositions. In plants, epimutations of endogenous genes have occasionally been described in laboratory strains, as with the alterations at the *P* locus of maize⁶ and at the *SUPERMAN* locus of *Arabidopsis*⁷. Studies on animals have not so far revealed heritable mutations of this kind, although epimutations have been recovered as somatic events in some cancers^{8,9}. One possible explanation is that, unlike the situation in animals, there is no early separation between germ line and soma in plants. Thus, an epimutation arising in a plant meristem can be transmitted to subsequent generations either by sexual means or through vegetative propagation. The mechanism for generating epimutations in plants is not known, but it may reflect aberrant activation within meristems of a process that can operate to silence genes in some non-meristematic cells. In the case of *Linaria*, which is an outbreeding perennial¹⁰, such epimutations may be more likely to underlie a natural mutant phenotype than DNA-sequence alterations which require two mutant alleles to come together to form a homozygote. This contrasts with laboratory populations where homozygosity is continually promoted by inbreeding and where the diminished role of vegetative propagation means there is less opportunity for somatic mutations to accumulate progressively in meristems.

Epimutations appear to be less stable than DNA-sequence alterations, as illustrated by the various degrees of somatic reversion in *Linaria*. Nevertheless, they may have longer-term consequences, depending on how the variation they cause interacts with variation generated by DNA-sequence changes. Furthermore, methylated DNA is more prone to mutation¹¹ and may influence the local frequency of recombination¹². Epimutations may therefore have both a short- and long-term significance for plant evolution^{13,14}. □

Methods

Plant material and crosses

Wild-type *Linaria vulgaris* plants were grown from seed (Unwins). Seeds were germinated on plates containing MS growth medium, grown in cabinets (at 20 °C for 16 h in light). Seedlings were then transplanted to pots and grown in a cool greenhouse. The peloric mutant was maintained by cuttings. For floral diagrams, more than ten flowers from different plants were examined for each phenotype. SEMs were carried out on plastic replicas as described¹⁵.

Five F_1 individuals were intercrossed (*Linaria* is self-incompatible) to generate eight F_2 segregating families. These generally contained few individuals owing to poor seed set and poor germination. In total, 39 F_2 individuals were obtained, of which 5 were fully peloric and the rest were wild-type. The segregations were (first number, wild type; second number, peloric): family 1: 16, 1; family 2: 1, 1; family 3: 1, 1; family 4: 5, 1; family 5: 0, 1; family 6: 6, 0; family 7: 4, 0; family 8: 1, 0. One of the peloric plants showed somatic instability during the first year, and three other peloric plants showed somatic instability after several years growth in the greenhouse. Seed capsules obtained from self-pollination of F_1 individuals showing a degree of self-compatibility gave 58 F_2 individuals, all of which were phenotypically wild type. This was possibly due to linkage of *Lcyc* to the self-incompatibility locus, as described in *Antirrhinum*^{16,17}.

DNA and RNA analysis

Genomic DNA was obtained from young leaves. DNA extraction and blot analysis was done as described¹⁸. The 3' end of the *Lcyc* transcript was isolated by RACE PCR: cDNAs were synthesized from wild-type *Linaria* mRNA as described¹⁹ and amplified by PCR²⁰ using an *Antirrhinum cycloidea*-specific oligonucleotide primer (5'-GAAAGTCTTTT-GATCTACA-3') from a very conserved region of the gene, the TCP domain²¹, together with oligonucleotide B25, which is complementary to the 3' end of the cDNA²⁰ (5'-GAC TCG AGT CGA CAT CGA-3'). PCR conditions were: 1 cycle at 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 45 °C for 2 min and 72 °C for 3 min, and a final step at 72 °C for 10 min. Seminested PCR was carried out on 1 µl of the previous PCR reaction with a further *cyc*-specific oligonucleotide nearer the 3' end (5'-GATGCTAGGTTTCGA-CAAGCCGAGCAAAACCCCTTG ATTGG-3'), together with B25 using similar conditions apart from annealing temperature, which was 50 °C instead of 45 °C. PCR products were gel-purified with Qiaquick (Qiagen), cloned in pGEM-T (Promega) and sequenced automatically (ABI system, Perkin Elmer). The genomic region flanking *Lcyc* was isolated by inverse PCR: 2–4 µg of wild-type and peloric genomic DNA was digested with *Hind*III, cleaned with a Wizard Clean-up kit (Promega), diluted fourfold, self-ligated with T4 ligase (Gibco) and used as a template for PCR, using oligonucleotides of *Lcyc* directed outwards from the gene (5'-ATGGAG TTGGATCTCGTTGCCG-3') and



Figure 5 Somatic instability of the peloric phenotype correlates with demethylation of *Lcyc*. **a**, Flowers from a peloric plant showing somatic instability. Arrows point to extra spurs on the lateral petals of the semipeloric flowers. The almost wild-type flower on the left has an extra dorsal stamen within the flower. **b**, DNA from young leaves of wild-type (+) and peloric (P) individuals digested and blotted as for Fig. 4b. The first two lanes corresponds to DNA from parental individuals; lane 3, DNA from an F_2 unstable mutant (U); lanes 4–6, DNA from cuttings from the unstable plant with peloric (P), semipeloric (SP) and near wild-type (+) phenotypes. Of the cuttings, only those with near wild-type phenotype show extensive demethylation of *Lcyc*. Based on the relative intensity of the 2.2-kb and 2.4-kb bands, semipelorics appeared to less heavily methylated than pelorics, consistent with their less extreme phenotype.

(5'-GTTTCGAAAGTCGCGAGGCGGC-3'). 1 µl from the reaction was used in a second PCR in which nested oligonucleotides further out (5'-ACACGGCAGGAGATTAA-GAGAGG-3' and 5'-AACT ACAGCAACGCATCTGCCCTCC-3') were used. PCR was carried out with an Expand Long Template PCR system (Boehringer Mannheim) under the conditions recommended by the manufacturer. Wild-type and peloric PCR products were cloned as pJAM2167 and pJAM2169, respectively, in pGEM-T (Promega) and sequenced. The 1.1-kb fragment used as probe A in the blots shown in Figs 3c, d and 4d was obtained by PCR carried out on wild-type genomic DNA and using oligonucleotides of *Lyc* pointing towards the gene (5'-TTTGGGAAGAACACATACC-3' and 5'-AGATCTTTGAGGAATGCAAAA GTTTTC-3'). The fragment was cloned as pJAM2166 in pGEM-T as described. Probe B (Fig. 4a) is a *HindIII/SacII* fragment obtained from pJAM2167. RFLP analysis of the F₂ families was carried out by digesting genomic DNA of the 39 individuals with *PstI*. The peloric phenotype showed complete linkage with absence of a 0.7-kb band. To analyse the segregation of a DNA polymorphism located 3' of the *Lyc* ORF, PCR was carried out on genomic DNA from four F₂ peloric plants and 16 F₂ wild types using oligonucleotides pointing towards the polymorphic region (5'-AAAGGTATT ATGAATAGTATATTAGTATTGG-3' and 5'-ATCTCATGAATTTTGATGTTAAAA-CATGATAGTAGC-3'). The resulting 290-bp fragments were purified with a Wizard clean-up kit (Promega) and automatically sequenced (ABI system, Perkin Elmer). The four peloric plants were homozygous for a G at position 2,228 (Fig. 3), like the peloric parental plant, whereas their wild-type siblings were either homozygous (A/A, 3 plants) like the wild-type parental plant or heterozygous (A/G, 13 plants). The four F₁ plants analysed were heterozygous (A/G). In the heterozygotes, two peaks, corresponding to two different nucleotides, were visualized at the polymorphic position.

The methods for digoxigenin labelling of RNA probes, tissue preparation and *in situ* hybridization have been described²².

Received 6 May; accepted 12 July 1999.

- Linnaeus, C. *De Peloria* (Diss. Ac. Amoenitates Academicæ III, Uppsala, 1749).
- Gustafsson, A. Linnaeus' peloria: the history of a monster. *Theor. Appl. Genet.* **54**, 241–248 (1979).
- De Vries, H. in *Species and Varieties: Their Origin by Mutations* 459–487 (Open Court, Chicago, 1904).
- Luo, D. et al. Origin of floral asymmetry in *Antirrhinum*. *Nature* **383**, 794–799 (1996).
- Almeida, J., Rocheta, M. & Galego, L. Genetic control of flower shape in *Antirrhinum majus*. *Development* **124**, 1387–1392 (1997).
- Das, O. P. & Messing, J. Variegated phenotype and developmental changes of a maize allele originating from epimutation. *Genetics* **136**, 1121–1141 (1994).
- Jacobsen, S. E. & Meyerowitz, E. M. Hypermethylated *SUPERMAN* epigenetic alleles in *Arabidopsis*. *Science* **277**, 1100–1103 (1997).
- Counts, J. L. & Goodman, J. I. Alterations in DNA methylation may play a variety of roles in carcinogenesis. *Cell* **83**, 13–15 (1995).
- Jones, P. A. & Laird, P. W. Cancer epigenetics comes of age. *Nature Genet.* **21**, 163–167 (1999).
- Docherty, Z. Self-incompatibility in *Linaria*. *Heredity* **49**, 349–352 (1982).
- Cambarelli, E. B., Jensen, B. C., Schabach, E. & Selker, E. U. Repeat-induced G-C to A-T mutations in *Neurospora*. *Science* **244**, 1571–1575 (1989).
- Hsieh, C.-L. & Lieber, M. R. CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication. *EMBO J.* **11**, 315–325 (1992).
- Jablonka, E. & Lamb, M. J. Epigenetic variation in evolution. *J. Evol. Biol.* **11**, 159–183 (1997).
- Jablonka, E. & Lamb, M. J. The inheritance of acquired epigenetic variations. *J. Theor. Biol.* **139**, 69–83 (1989).
- Williams, M. H. & Green, P. B. Sequential scanning electron microscopy of a growing plant meristem. *Protoplasma* **147**, 77–79 (1988).
- Brieger, F. G. The inheritance of self-sterility and the peloric flower shape in *Antirrhinum*. *Genetics* **17**, 385–408 (1935).
- Sherman, M. The inheritance of self-sterility in certain species of *Antirrhinum*. *Z. Indukt Abstammungs-Vererbungs* **77**, 1–17 (1939).
- Bradley, D. et al. Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the *plena* locus of *Antirrhinum*. *Cell* **72**, 85–95 (1993).
- Gubler, U. A one tube reaction for the synthesis of blunt-ended double-stranded cDNA. *Nucleic Acids Res.* **16**, 2726 (1988).
- Frohman, M. A., Dush, M. K. & Martin, G. R. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl Acad. Sci. USA* **85**, 8998–9002 (1988).
- Cubas, P., Lauter, N., Doebley, J. & Coen, E. The TCP domain: a motif found in proteins regulating plant growth and development. *Plant J.* **18**, 215–222 (1999).
- Coen, E. et al. *Floricula*: a homeotic gene required for flower development in *Antirrhinum majus*. *Cell* **63**, 1311–1322 (1990).
- Carpenter, R. et al. Control of flower development and phyllotaxy by meristem identity genes in *Antirrhinum*. *The Plant Cell* **7**, 2001–2011 (1995).
- Bradley, D. et al. Control of inflorescence architecture in *Antirrhinum*. *Nature* **379**, 791–797 (1996).

Acknowledgements

We thank the Linnean Society of London for permission to photograph the specimen of peloric *Linaria* kept in Linnaeus' herbarium and thank C. Jarvis from the Natural History Museum in London for providing the photograph; we also thank M. Cragg-Barber for providing a living peloric specimen from the UK; N. Hartley for sequencing the genomic *Lyc* region; D. Bradley for the *Lcentroradialis* probe; C. Martin for the *Antirrhinum ubiquitin* probe; and R. Carpenter, D. Bradley, O. Ratcliffe, I. Amaya and U. Nath for comments on the manuscript. This work was supported by the Gatsby Charitable Foundation. P.C. was an EMBO postdoctoral fellow and a EU postdoctoral fellow.

Correspondence and requests for materials should be addressed to E.C. (e-mail: coenen@bbsrc.ac.uk). The Genbank accession number for the *Lyc* sequence is Bank AF 161252.

A role for *Gbx2* in repression of *Otx2* and positioning the mid/hindbrain organizer

Sandrine Millet^{††}, Kenneth Campbell^{*†‡}, Douglas J. Epstein^{†‡}, Kasia Losos[†], Esther Harris[†] & Alexandra L. Joyner^{†§}

[†] Developmental Genetics Program and Howard Hughes Medical Institute, Skirball Institute of Biomolecular Medicine, § Department of Cell Biology and Physiology and Neuroscience, NYU School of Medicine, 540 First Avenue, New York, New York 10016, USA

* These authors contributed equally to this work.

The mid/hindbrain (MHB) junction can act as an organizer to direct the development of the midbrain and anterior hindbrain^{1,2}. In mice, *Otx2* is expressed in the forebrain and midbrain and *Gbx2* is expressed in the anterior hindbrain, with a shared border at the level of the MHB organizer. Here we show that, in *Gbx2*^{-/-} mutants, the earliest phenotype is a posterior expansion of the *Otx2* domain during early somite stages. Furthermore, organizer genes are expressed at the shifted *Otx2* border, but not in a normal spatial relationship. To test whether *Gbx2* is sufficient to position the MHB organizer, we transiently expressed *Gbx2* in the caudal *Otx2* domain and found that the *Otx2* caudal border was indeed shifted rostrally and a normal appearing organizer formed at this new *Otx2* border. Transgenic embryos then showed an expanded hindbrain and a reduced midbrain at embryonic day 9.5–10. We propose that formation of a normal MHB organizer depends on a sharp *Otx2* caudal border and that *Gbx2* is required to position and sharpen this border.

Otx2 null mutants have a deletion of the brain rostral to hindbrain rhombomere 3 (r3), due to a failure of induction of the anterior neural plate during gastrulation^{3–5}. *Otx1* mutants have only subtle defects⁶. In double *Otx1/Otx2* mutants^{7,8} that have only one *Otx2* wild-type allele, the *Otx2* caudal limit and the MHB organizer are shifted anteriorly at early somite stages (ESS). Subsequently, no mesencephalon (midbrain) and caudal forebrain form and the cerebellum (normally arising from the anterior hindbrain or metencephalon) is expanded rostrally. In contrast, *Gbx2* null mutants lack the rostral hindbrain and have a caudal expansion of the midbrain at E12.5, and have abnormalities in the MHB organizer at E9.5 (ref. 9).

To investigate the specific role of *Gbx2* in formation of the MHB organizer, we reanalysed MHB gene expression in *Gbx2* mutants at ESS. At 4–6 somites in these mutants, the *Otx2* domain was clearly expanded and the posterior limit shifted caudally from the middle of the MHB region to the r3/4 border (Fig. 1a–d). Furthermore, the *Otx2* limit did not sharpen in the mutant embryos (Fig. 1c, d). At ESS, the domain of expression of the organizer gene *Fgf8* was expanded and shifted caudally (Fig. 1e–h) from r2 to r4 in *Gbx2* mutants, and the gradient of expression was inverted with the strongest expression in r4 (Fig. 1h). The normal expression pattern of the organizer gene *Wnt1* at ESS (Fig. 1i, k) can be subdivided into a domain of expression along the lateral edges (future dorsal midline) between the diencephalon and the MHB junction and caudally from r4 into the spinal cord, and a domain of expression in the mesencephalon. In *Gbx2* mutants, the expression along the lateral edges was continuous without a negative gap in the metencephalon (Fig. 1j, l), indicating that *Gbx2* could be required to repress *Wnt1* in this region. The *Wnt1* domain in the mesencephalon was slightly expanded and clearly shifted caudally, so that it was

[‡] Present addresses: Wallenberg Neuroscience Center, Lund University, Sölvegatan 17, S-223 62 Lund, Sweden (K.C.); Department of Genetics, University of Pennsylvania Medical Center, Clinical Research Bldg., Room 463, 415 Curie Boulevard, Philadelphia, Pennsylvania 19104-6145, USA (D.J.E.).

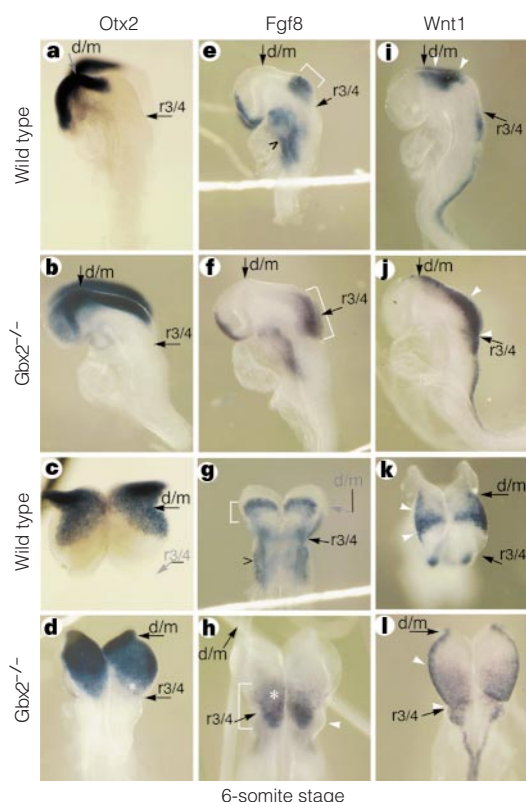


Figure 1 Transformation of the metencephalon towards a mesencephalic fate at ESS in *Gbx2* mutants. Expression of *Otx2* (a–d), *Fgf8* (e–h) and *Wnt1* (i–l) in wild-type (first and third rows) and *Gbx2* mutant (second and fourth rows) embryos at the 6-somite stage. Two upper rows: sagittal view, rostral on left; two bottom rows: dorsal view, rostral on top. Asterisks show fainter expression; r3/4, rhombomere 3/4 boundary; d/m, mes/diencephalic boundary; brackets in e–h and white arrowheads in i–l, limits of *Fgf8* and *Wnt1* expression, respectively, in the MHB region. Note that the more caudal and ventral staining marked by > in g and e corresponds to mesodermal expression.

fused with the expression in r4 (Fig. 1j, l). However, the caudal-to-rostral decreasing gradient was preserved. Thus, the earliest detectable phenotype observed in *Gbx2* mutants is a respecification of the anterior hindbrain, and not a deletion.

The *Gbx2* mutant analysis indicates that *Gbx2* is involved in positioning the MHB organizer by repressing *Otx2* at ESS. To test this hypothesis, we transiently expressed *Gbx2* in the midbrain at this stage under the control of a *Wnt1* enhancer¹⁰ and analysed the resulting phenotype either in primary embryos directly injected with the construct or in embryos from a *Wnt1-Gbx2* transgenic line. Analysis of *Wnt1-Gbx2* embryos at the 6-somite stage showed that *Gbx2* was ectopically expressed across the mesencephalon and along the lateral edges (Fig. 2a–d). Consistent with our hypothesis, the *Otx2* caudal limit was shifted rostrally in the mesencephalon of *Wnt1-Gbx2* embryos (Fig. 2e–h). The exact location of the new *Otx2* limit varied between transgenics, but it was always sharp and located within the rostral mesencephalon. The fact that in transgenics the *Otx2* domain stopped abruptly within a territory weakly expressing *Gbx2* indicates that there may be a minimum threshold of *Gbx2* required to repress *Otx2* expression in an all-or-none manner. This latter characteristic could provide a basis for the mechanism by which *Gbx2* sharpens the *Otx2* caudal limit and positions the MHB organizer.

The *Wnt1* and *Fgf8* domains were also shifted rostrally in 6–10-somite-stage transgenic embryos (Fig. 2i–p), with their relative positions and gradients remaining normal and the *Wnt1* domain appearing shortened. A *lacZ* probe that detects the *Wnt1-Gbx2* transgene did not detect transcripts in the caudal mesencephalon (Fig. 2q), whereas the *Gbx2* probe detected expression in the entire

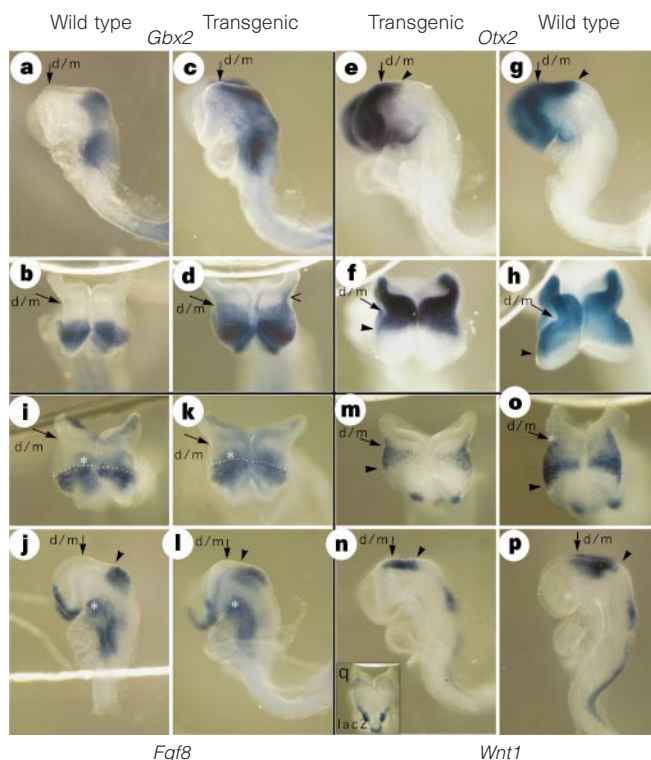


Figure 2 *Otx2* and *Wnt1* are repressed and *Gbx2* and *Fgf8* are induced in the caudal midbrain at ESS in *Wnt1-Gbx2* transgenics. Expression of *Gbx2* (a–d), *Otx2* (e–h), *Fgf8* (i–l), *Wnt1* (m–p) and *lacZ* (q) in wild-type (a, b, g–j, o, p) and *Wnt1-Gbx2* transgenic (c–f, k–n, q) embryos at the 6-somite stage. Top and bottom rows: sagittal view, rostral on left; middle rows: dorsal view at the level of the mes/diencephalic boundary (d/m), rostral on top. *Gbx2* expression along the lateral edges is faint and variable rostral to d/m (< in d) and stronger caudal to r4 (not shown in the plane of focus). e–p, Arrowheads point to the *Otx2*, *Fgf8* or *Wnt1* border in the MHB junction. The rostral limit of the *Fgf8* staining in the MHB region is indicated by the white dashed line (i, k). The staining indicated by asterisks corresponds to *Fgf8* expression in the underlying mesoderm (j, l).

mesencephalon and metencephalon (Fig. 2c, d). This indicates that endogenous *Gbx2* was induced in the caudal mesencephalon.

At E9.5, 7 embryos, out of 15 primary transgenic embryos obtained, expressed the transgene. As expected, based on the gene-expression studies at ESS, all displayed, with varying degrees of severity, a decrease in the size of the midbrain as well as a slight expansion of the metencephalon based on marker-gene expression (*Otx2*, *Meis2*, *Hoxb1*, *Pax6* and *En1*) and the position of the MHB constriction (Fig. 3; and data not shown). All of the homozygous transgenic embryos collected showed a moderate MHB alteration (Fig. 3). It appears that a rostral portion of the mesencephalon was the most severely compromised at E9.5, as the size of the *En1* expression domain at the MHB junction in homozygous transgenics appeared normal, whereas the size of the *En1/Pax6*-negative domain in the rostral mesencephalon was reduced (Fig. 3c, f). Despite the altered morphology in the *Wnt1-Gbx2* embryos at E9.5, *Wnt1* and *Fgf8* expression seemed to retain a normal spatial relationship with respect to the *Otx2* caudal limit (Fig. 3g–i; and data not shown). At E9.5 the transgene was not regulated in the same way as the endogenous *Wnt1* gene (Fig. 3j–l), as *lacZ* expression was barely detectable in the MHB junction but was obvious along the dorsal midline outside the MHB region. As such a difference is not observed when *lacZ* is expressed from the *Wnt1* promoter¹⁰, this indicates that *Gbx2* directly or indirectly inhibits the transgene in the MHB region.

Following the downregulation of the transgene, by E12.5 the MHB region of *Wnt1-Gbx2* transgenic embryos appeared normal (Fig. 3m, n). Brain sections of homozygous newborn and adult

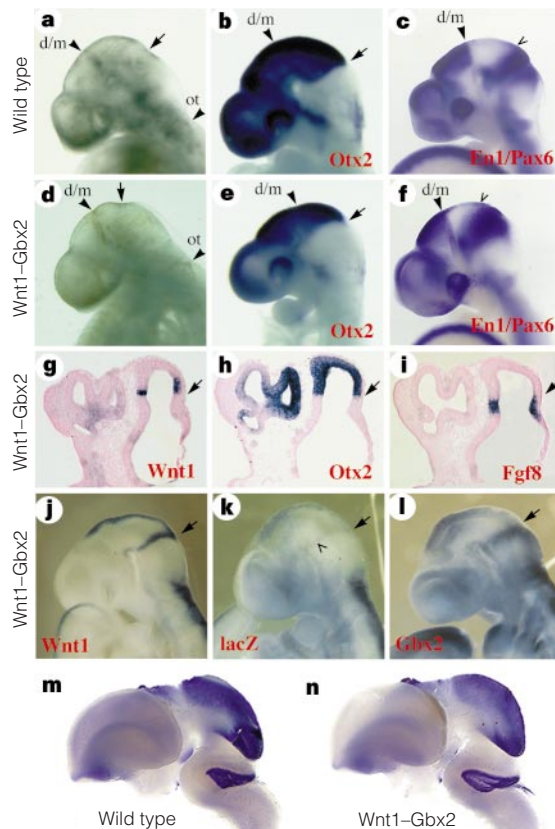


Figure 3 The sizes of the mesencephalon and metencephalon are altered transiently in *Wnt1-Gbx2* transgenics. Sagittal view of wild-type (**a–c, m**) and *Wnt1-Gbx2* transgenic embryos (primary transgenics: **d, e**; homozygote *Wnt1-Gbx2*: **f–l, n**) at E9.5 (**a–l**) and E12.5 (**m, n**). Rostral is on the left. Whole-mount embryos unstained (**a, d**) or stained with *Otx2* (**b, e, m, n**), *En1* and *Pax6* (**c, f**), *Wnt1* (**j**), *lacZ* (**k**) or *Gbx2* (**l**). Adjacent cryostat sections stained with *Wnt1* (**g**), *Otx2* (**h**) or *Fgf8* (**i**). Arrows in **a, b, d, e, g–i** point to the MHB junction. Arrowheads point to the *En1* rostral limit in **c, f** and to the very faint *lacZ* expression in the MHB junction in **k, d, m**, mes/diencephalic boundary; ot, otic vesicle.

Wnt1-Gbx2 transgenics showed no obvious morphological abnormalities. As endogenous *Gbx2* expression was induced at E8.5 in the caudal mesencephalon of the transgenics, when the transgene is later downregulated a new equilibrium must be established between *Otx2* and *Gbx2*. The recovery of normal proportions between the mesencephalon and metencephalon by E12.5 could reflect a progressive posterior shift in the *Otx2/Gbx2* border or a regulation of the size of the two compartments. The rostral shift of the organizer when *Gbx2* is ectopically expressed and the rescue following the downregulation of this transgene clearly show the crucial role of *Gbx2* in positioning the organizer.

In conclusion, we provide two kinds of genetic evidence that *Gbx2* is normally involved in positioning the MHB organizer, by directly or indirectly repressing *Otx2* in the caudal portion of the MHB region (Fig. 4). Based on *Otx1/2* double-mutant studies^{7,8}, there may be a reciprocal repression of *Gbx2* by *Otx2*. A mutual repression between *Otx2* and *Gbx2* at ESS could establish an equilibrium between these genes at the MHB junction, resulting in sharpening and positioning of the *Otx2/Gbx2* border. The juxtaposition of molecularly distinct territories could then induce the molecular cascade underlying the MHB organizer. A second role for *Gbx2* appears to be in maintaining an *Otx2*-negative territory from which the cerebellum can develop. In *Gbx2* mutants, r4 is not transformed into a cerebellum⁹, although it is located immediately caudal to *Otx2* and expresses *Fgf8*. As, in chick/quail transplantation experiments, the whole hindbrain is competent to be respecified into cerebellum by a transplanted organizer¹¹, one possibility is that

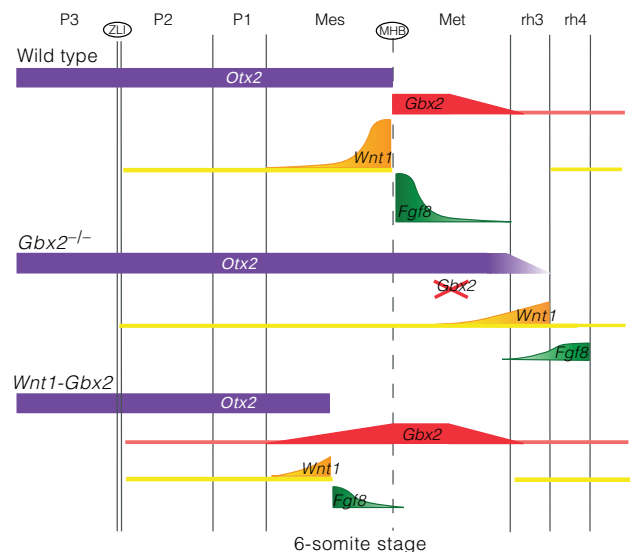


Figure 4 Schematic representation of the *Otx2*, *Gbx2*, *Wnt1* and *Fgf8* domains of expression in wild-type, *Gbx2* mutant and *Wnt1-Gbx2* transgenic embryos at the 6-somite stage. The thickness of the bars is proportional to the level of gene expression. Orange represents the mesencephalic expression of *Wnt1* and yellow the lateral expression. ZLI, zona limitans intrathalamica; MHB, MHB junction; mes, mesencephalon; met, metencephalon; P1–P3, diencephalic prosomeres 1 to 3 (nomenclature of ref. 19).

additional factors required for cerebellum development, possibly *Gbx2* itself, are missing in r4 of *Gbx2* mutants. Conditional inactivation of *Gbx2* will be required to determine the sequential functions of *Gbx2*. □

Methods

Generation and maintenance of transgenic mice

Gbx2 mutant mice⁹ were genotyped by PCR on tail or yolk-sac DNA, as described⁹.

We prepared the *Wnt1-Gbx2* transgenic construct by blunt ligating a 1050-base pair (bp) *HindIII*–*Acc65I* fragment containing the coding region of the mouse *Gbx2* complementary DNA (nucleotides 430–1480; provided by M. Frohman) into the *EcoRV* site of the pWexp3 construct¹⁰ to generate *Wnt1-Gbx2*. CD-1 transgenic embryos and mouse lines were generated as described¹². Transgenic embryos and mice were genotyped using PCR with primers specific for the *lacZ* tag (sense: 5'-CCGAACCATCCGCTGTGGTAC-3'; antisense: 5'-CATCCACGCGCGGTACATC-3') or Southern blot analysis of *EcoRI*-digested DNA with a *lacZ* probe. We monitored expression of the transgene construct by *in situ* hybridization with a *lacZ* probe.

In situ hybridization

For transient *Wnt1-Gbx2* transgenic experiments, the day of transfer into the pseudo-pregnant mother was designated E1.5. Timed *Wnt1-Gbx2* embryos were obtained from matings of *Wnt1-Gbx2* hemi- or homozygous mice together or with wild-type CD-1 or SW mice and *Gbx2* homozygous embryos from interbreeding *Gbx2* heterozygous mice. The day on which the vaginal plug was detected was designated E0.5. At the desired stages, embryos were fixed overnight in 4% paraformaldehyde at 4°C, rinsed in PBS and dehydrated in methanol. Whole-mount and section *in situ* hybridizations were carried out essentially as described^{13,14}. The probes used were *Otx2* (J. Rossant), *Gbx2* (M. Frohman), *Fgf8* (L. Niswander), *Wnt1* (ref. 15), *En1*, *En2* (ref. 16), *lacZ* (A. P. McMahon), *Pax6* (B. Hogan), *Hoxb1* (ref. 17) and *Meis2* (ref. 18).

Received 26 April; accepted 8 July 1999.

- Wassef, M. & Joyner, A. L. Early mesencephalon/metencephalon patterning and development of the cerebellum. *Perspect. Dev. Neurobiol.* **5**, 3–16 (1997).
- Alvarado-Mallart, R. M. Fate and potentialities of the avian mesencephalic/metencephalic neuroepithelium. *J. Neurobiol.* **24**, 1341–1355 (1993).
- Acampora, D. et al. Forebrain and midbrain regions are deleted in *Otx2*–/– mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121**, 3279–3290 (1995).
- Matsuo, I., Kuratani, S., Kimura, C., Takeda, N., Aizawa, S. Mouse *Otx2* functions in the formation and patterning of rostral head. *Genes Dev.* **9**, 2646–2658 (1995).
- Rhinn, M. et al. Sequential roles for *Otx2* in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* **125**, 845–856 (1998).
- Acampora, D. et al. Epilepsy and brain abnormalities in mice lacking *Otx1* gene. *Nature Genet.* **14**, 218–222 (1996).
- Acampora, D., Avantsaggiato, V., Tuorto, F. & Simeone, A. Genetic control of brain morphogenesis through *Otx* gene dosage requirement. *Development* **124**, 3639–3650 (1997).

8. Suda, Y., Matsuo, I. & Aizawa, S. Cooperation between *Otx1* and *Otx2* genes in developmental patterning of rostral brain. *Mech. Dev.* **69**, 125–141 (1997).
9. Wassermann, K. M. *et al.* Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function. *Development* **124**, 2923–2934 (1997).
10. Echelard, Y., Vassileva, G. & McMahon, A. P. Cis-acting regulatory sequences governing *Wnt1* expression in the developing mouse CNS. *Development* **120**, 2213–2224 (1994).
11. Martinez, S., Marin, F., Nieto, M. A. & Puellas, L. Induction of ectopic *engrailed* expression and fat change in avian rhombomeres: intersegmental boundaries as barriers. *Mech. Dev.* **51**, 289–303 (1995).
12. Hogan, B., Beddington, R., Constantini, F. *Manipulating the Mouse Embryo. A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1994).
13. Wilkinson, D. *In Situ Hybridization. A Practical Approach* (IRL, Oxford, 1992).
14. Schaefer-Wiemers, N. & Gerfin-Moser, A. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* **100**, 431–440 (1993).
15. McMahon, A. P., Joyner, A. L., Bradley, A. & McMahon, J. A. The midbrain-hindbrain phenotype of *Wnt1*/*Wnt1*-mice results from stepwise deletion of *engrailed*-expressing cell by 9.5 days postcoitum. *Cell* **69**, 581–595 (1992).
16. Millen, K. J., Hui, C. C. & Joyner, A. L. A role for *En-2* and other murine homologues of *Drosophila* segment polarity genes in regulating positional information in the developing cerebellum. *Development* **121**, 3935–3945 (1995).
17. Frohman, M. A., Boyle, M. & Martin, G. Isolation of the mouse *Hox-2.9* gene: analysis of embryonic expression suggests that positional information along the anterior posterior axis is specified by mesoderm. *Development* **110**, 589–607 (1990).
18. Toresson, H., Mata de Urquiza, A., Fragerstrom, C., Perlmann, T. & Campbell, K. Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation. *Development* **126**, 1317–1326 (1999).
19. Puellas, L. & Rubenstein, J. L. R. Expression of homeobox and other putative regulatory genes in the mouse forebrain suggest a neuromeric organization. *Trends Neurosci.* **16**, 472–479 (1993).

Acknowledgements

We thank A. Liu and Y. Li for helpful discussions and comments on the manuscript, and the Skirball transgenic facility for assistance with transgenic production. This work was supported by grants from the Human Frontiers Science Program (HFSP) and NINDS. S.M. was supported by an HFSP fellowship, and K.C. and D.J.E. by Canadian MRC fellowships. A.L.J. is an HHMI investigator.

Correspondence and requests for materials should be addressed to A.L.J. (e-mail: joyner@saturn.med.nyu.edu).

The caudal limit of *Otx2* expression positions the isthmic organizer

Vania Broccoli¹*, Edoardo Boncinelli² & Wolfgang Wurst¹†

¹ GSF-Research Centre for Environment and Health, Institute of Mammalian Genetics, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany

² Max Planck Institute of Psychiatry, Kraepelinstrasse 2-16, D-80804 Munich, Germany

§ DIBIT-HSRaffaele, Via Olgettina 58, 20132 Milan, Italy

The homeobox gene *Otx2* is expressed in the anterior neural tube with a sharp limit at the midbrain/hindbrain junction (the isthmic organizer)¹. *Otx2* inactivation experiments have shown that this gene is essential for the development of its expression domain². Here we investigate whether the caudal limit of *Otx2* expression is instrumental in positioning the isthmic organizer and in specifying midbrain versus hindbrain fate, by ectopically expressing *Otx2* in the presumptive anterior hindbrain using a knock-in strategy into the *En1* locus. Transgenic offspring display a cerebellar ataxia. Morphological and histological studies of adult transgenic brains reveal that most of the anterior cerebellar vermis is missing, whereas the inferior colliculus is complementarily enlarged. During early neural pattern formation expression of the midbrain markers *Wnt1* and *Ephrin-A5*, the isthmic organizer markers *Pax2* and *Fgf-8* and the hindbrain marker *Gbx2* are shifted caudally in the presumptive hindbrain territory. These findings show that the caudal limit of *Otx2* expression is

sufficient for positioning the isthmic organizer and encoding caudal midbrain fate within the mid/hindbrain domain.

Early neural-plate pattern formation is determined by exogenous signals from the mesoderm and visceral endoderm, and from planar signals active within the neuroectoderm^{3,4}. The combination of these signals influences region-specific gene expression, for instance restricting *Otx2* to the anterior neural plate (presumptive forebrain and midbrain) and *Gx2* to the posterior neural plate (presumptive hindbrain and spinal cord)^{4–6}. Subsequently, signalling molecules such as *Fgf-8* and *Wnt1* are expressed on either side of the mid/hindbrain border, controlling patterning and growth of adjacent territories^{7,8}. This border acts like an organizing centre (the isthmic organizer). It can induce and reconstruct a mid/hindbrain domain in its vicinity when it is grafted ectopically into the forebrain or hindbrain^{9–13}. *Fgf-8* can mimic this effect of the isthmic organizer when released from acrylic beads transplanted into similar positions⁸. It has been postulated that the establishment of such

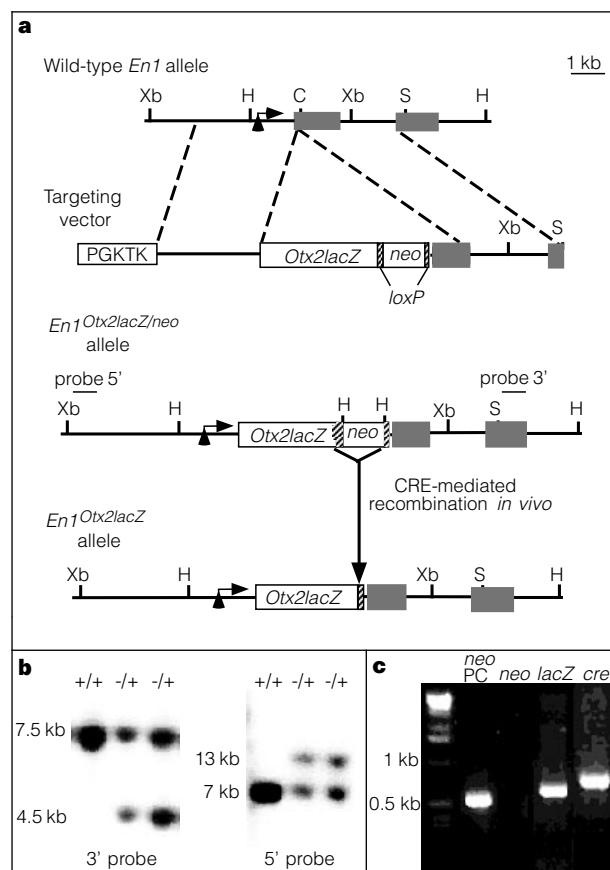


Figure 1 Knock-in strategy of *Otx2IRESlacZ* (*Otx2lacZ*) into the *En1* locus. **a**, Arrow, *En1* promoter; grey boxes, coding sequences flanking a single intron. The *En1*^{*Otx2lacZ*} targeting vector contains *Otx2lacZ* cassette, selectable thymidine kinase (TK) and a neomycin gene flanked by *loxP* sites. Dashed lines show regions of identity between the locus and the targeting vector. After gene targeting, *Otx2lacZ* is inserted into the *En1* locus downstream of the promoter, deleting the first 111 amino acids of *En1* and generating a null mutation. The positions of the 5' and 3' probes used to verify homologous recombination are indicated. After *cre* recombinase activation, the neomycin selector gene was removed leaving a single *loxP* site (shaded box). C, *Clal*; H, *HindIII*; S, *Sad*; Xb, *XbaI* restriction enzymes. **b**, Southern blot of genomic DNA isolated from targeted (+/-) ES cells. Digestion with *HindIII* and hybridization with a 3' probe (left panel) revealed an additional 4.5-kb fragment; digestion with *XbaI* and hybridization with a 5' probe (right panel) revealed an additional 13-kb fragment, indicative of a proper targeting event. **c**, After germline transmission the F1 generation was bred with a *cre* deleter strain to remove the *neo* cassette. Using specific primer sets recognizing *neomycin*, *lacZ* and *cre*, we show that the offspring expressing *cre*, recombinase have lost the *neomycin* gene (right three lanes, *neo* PC represents the *neo* control).

† Present address: Telethon Institute of Genetics and Medicine (TIGEM), San Raffaele Biomedical Science Park, 20132 Milan, Italy.

organizing centres first requires the specification of two different cell populations in adjacent territories¹⁴. At the presumptive mid/hindbrain border, the juxtaposed expression of *Otx2* and *Gbx2* could perform this function.

To determine whether the sharp caudal limit of *Otx2* expression at the mid/hindbrain border is indeed critical for the formation and localization of the isthmus organizer and specification of midbrain versus hindbrain fate, we ectopically shifted the caudal limit of *Otx2* expression across the mid/hindbrain border into the anterior hindbrain (into the rostral third of rhombomere 1, rh1) by replacing the coding sequence of *En1* with *Otx2* (knock-in approach) using homologous recombination in embryonic stem (ES) cells (Fig. 1a). To allow us to follow easily the ectopic expression of *Otx2*, an internal ribosomal entry site (IRES) containing the *lacZ* gene was included. Thus, *Otx2* and *lacZ* were under the control of the *En1* regulatory elements and were expressed ectopically in the caudal midbrain and the anterior hindbrain domain of *En1* (ref. 15). From 300 recombinant ES cell colonies tested, we obtained 21 colonies carrying the designed modification (Fig. 1b). Germline chimaeras were bred to C57B1/6 or 129/Sv strains to obtain F1 offspring. The F1 animals were crossed with a transgenic line constitutively expressing *cre* recombinase to remove the PGK-promoter neomycin cassette, which might influence *Otx2* expression (Fig. 1c)¹⁶. Indeed, the expression of *Otx2* was about 2-fold higher after PGK-*neo* removal (data not shown).

Adult F1 heterozygote mutants were readily identified by their altered motor behaviour. They exhibited a gait ataxia and a wide stance. In simple motor behaviour tests such as the stationary rotarod ($n = 6$), horizontal thin rod ($n = 6$) and running rotarod ($n = 6$), the mutants performed significantly less well than wild-type littermates. The performance of the mutants improved slightly

on the stationary rod and horizontal thin rod after six consecutive days of testing, but not on the running rotarod (see Supplementary Information). To measure muscle strength, a wire suspension test was applied, on which mutants and wild-type mice performed equally well (data not shown), indicating that the differences observed in the motor behaviour paradigms are due to cerebellar dysfunction.

We assessed the effect of ectopic expression of *Otx2* on cerebellar development in 12-week-old animals or at E15 by histology, immunohistochemistry and *in situ* hybridization of brain sections or whole mounts. There were clear morphological differences. The superior and inferior colliculi appeared enlarged and were shifted posteriorly in all mutants analysed, and the vermis of the cerebellum was reduced in size and not fused in the midline in *En1^{+/Otx2lacZ}* transgenic mice (Fig. 2b), unlike in *En1^{+/+}* and wild-type mice (Fig. 2a). The inferior colliculi of mutants were enlarged and significantly heavier (mean 18.81 mg $\pm$ 0.26, $n = 6$) than those of wild-type mice (14.01 mg $\pm$ 0.183, $n = 6$, $P < 0.005$, *U*-test). In the cerebellum, most of the anterior vermis was missing in the mutants (Fig. 2d). Unlike the wild-type (Fig. 2c), only the posterior cerebellar lobules VIII, IX and X were present in mutants, but they were not fused in the midline (Fig. 2d). They exhibited longitudinal instead of transverse fissures (Fig. 2c, d). The two longitudinal fissures that normally separate the central vermis from the lateral hemisphere were also missing (Fig. 2d). An additional lobe had formed in the cerebellar hemisphere, between crusII and the paramedial lobe (Fig. 2d). Coronal sections of adult wild-type (Fig. 2e) and mutant (Fig. 2f) cerebellum confirmed the phenotypic alterations observed in the whole-mount cerebellum. In coronal sections, using Zebrin II as a marker for longitudinal compartmentalization of the vermis and hemispheres, we confirmed the loss of anterior vermis tissue and altered foliation patterns in the hemispheres (Fig. 2e, f; and data not shown). The cerebellar commissure, which is normally located within the inner granular cell layer of the anterior cerebellum, was

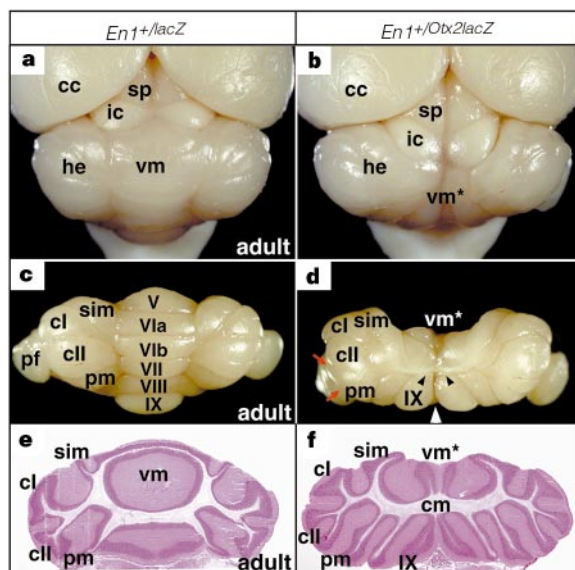


Figure 2 Ectopic expression of *Otx2* results in phenotypic alterations of midbrain and cerebellum. **a, b**, Dorsal view of brains of adult wild-type (*En1^{+/lacZ}*, **a**) and *En1^{+/Otx2lacZ}* transgenic mice (**b**) show the caudal shift of the superior and inferior colliculi, the loss of most of the anterior vermis and the lack of cerebellar midline fusions in the mutants. **c, d**, Dorsal view of the dissected cerebellum shows deletion of most of the anterior vermis in the mutant (**d**) compared with the wild-type (**c**). Lobes VIII, IX and X (hidden) of the posterior vermis are present but not fused in the midline (white arrowhead). The cerebellar commissure appears at the surface (black arrowheads). The foliation patterns of the cerebellar hemispheres are altered in transgenic mice with an additional lobe between crusII and the paramedial lobe (red arrows). **e, f**, Coronal sections of wild-type (**e**) and mutant (**f**) cerebellum confirm these interpretations. cc, cerebral cortex; cb, cerebellum; cl, crus I; cll, crus II; he, hemisphere; ic, inferior colliculi; is, isthmus; pf, paraflocculus; pm, paramedial lobe; sim, simplex; sp, superior colliculi; vm, vermis; vermis lobes V–IX are indicated.

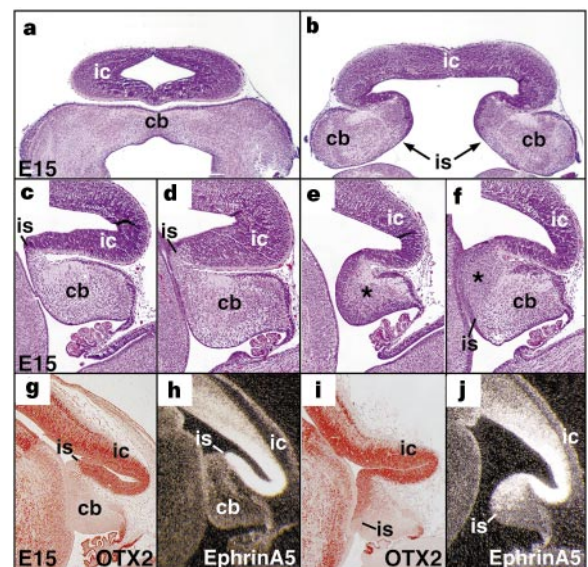


Figure 3 Ectopic expression of *Otx2* results in phenotypic alterations at E15. Coronal sections of wild-type (**a**) and mutant brain (**b**) at the level of the inferior colliculi at stage E15. In the mutants, the bilateral cerebellar anlagen are not fused in the midline and inferior colliculi and isthmus cells are located in the cerebellar anlage (arrow). **c–f**, Sagittal sections of wild-type (**c, d**) and mutant (**e, f**) brains at E15. The densely packed inferior colliculi cells and the loosely packed isthmus cells (**c, d**) are positioned in the anterior cerebellum in the mutants (**e, f**; star). **g, i**, Anti- α -Otx2 antibody staining on wild-type (**g**) and mutant (**i**) sagittal brains at E15 indicate extension of *Otx2* expression into the cerebellar anlage. *In situ* hybridization of sagittal sections using *Ephrin-A5* in wild-type (**h**) and mutant (**j**) brains at E15 reveal *Ephrin-A5*-positive cells in the cerebellar anlage in the mutant animals.

displaced ectopically to the surface of the posterior vermis (Fig. 2d). Despite the morphological alterations, the cytoarchitecture of the mutant cerebellum was normal.

At E15, the tectal and lateral cerebellar primordia fused at the midline in the wild-type brains, as shown in coronal sections (Fig. 3a). In contrast, in the mutants the lateral cerebellar anlagen did not fuse in the midline (Fig. 3b), and compact, intensely stained inferior-colliculi-specific and isthmus-specific cells were observed in the anterior cerebellum (compare Fig. 3b, e, f with 3c, d). To confirm that these anterior transformations were due to ectopic expression of *Otx2*, we studied *Otx2* expression at different embryonic stages by mRNA *in situ* hybridization and antibody staining. As shown in Fig. 3i, OTX2 was expressed ectopically in the cerebellum in an *En1*-like manner, confirming the histological observations. Furthermore, using *Ephrin-A5* as a midbrain-specific marker (Fig. 3h; see also Supplementary Information), we detected midbrain-specific cells in the mutant anterior cerebellum (Fig. 3j). These findings agree with fate-map studies performed by chick/quail transplantations, which indicated that the early embryonic, caudal *Otx2* expression domain will give rise to midbrain and the *Otx2*-negative domain in the presumptive isthmus region and anterior rhombomere 1 to the cerebellar vermis in a V-shaped manner^{17,18}. To investigate whether these mid/hindbrain transformations are a consequence of repositioning of the isthmus organizer in the rostral hindbrain, we examined E12, E10.5 and E9.5 embryos for isthmus organizer formation and

phenotypic alterations using different molecular markers.

Whole-mount *in situ* hybridization at E10.5 in mutant embryos showed that the expression of *Otx2* (Fig. 4b, g), *Pax2* (Fig. 4d, h) and *En1* (Fig. 4j) were shifted caudally into the anterior rh1 domain. At E9.5, *Otx2* (Fig. 5b), *Wnt1* (Fig. 5d), *Fgf-8* (Fig. 5f) and *Ephrin-A5* (not shown) were induced ectopically in the dorsal roof of the hindbrain, whereas *Gbx2* (Fig. 5h) was repressed in the ectopic *Otx2* expression domain (Fig. 5b). These ectopic inductions and repressions were all maintained at E12 (see Supplementary Information). *En2*, which is expressed across the mid/hindbrain junction in a broad domain, seems unaltered (not shown); however, subtle changes within the *En2* expression domain cannot be ruled out, as they would be difficult to visualize. Thus, at early embryonic stages, the expression of all the midbrain- (*Ephrin-A5*, *Wnt1*) and isthmus-specific markers (*Pax2*, *Fgf-8*) was shifted caudally into rh1 as a consequence of the ectopic *Otx2* expression, whereas the hindbrain-specific marker *Gbx2* was repressed. As a result, the isthmus organizer was repositioned in the anterior hindbrain during early development, with the inferior colliculi enlarged and extended caudally at the expense of the anterior vermis in the adult.

Loss-of-function and gain-of-function experiments in mouse,

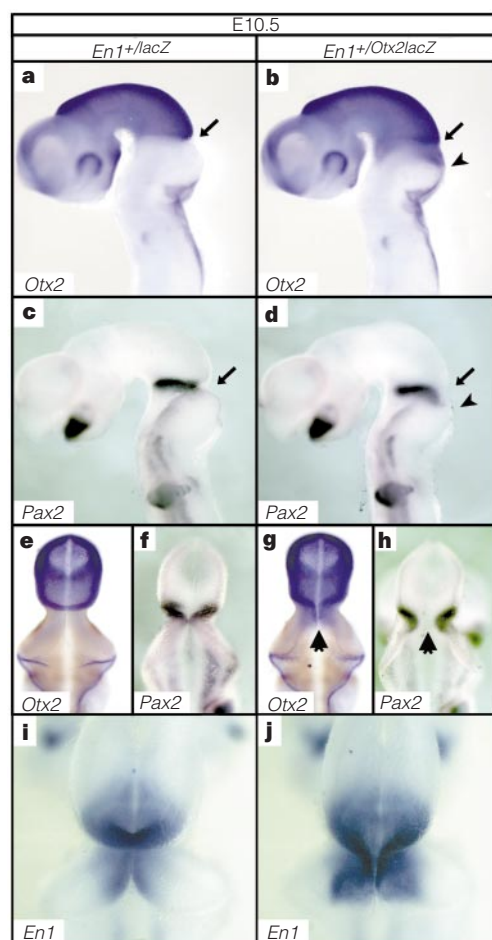


Figure 4 mRNA *in situ* hybridization of mid/hindbrain-specific markers in wild-type (*En1*^{+/lacZ}; **a, c, e, f, i**) and mutants (*En1*^{+/Otx2lacZ}; **b, d, g, h, j**) in a lateral (**a–d**) and dorsal view (**e–j**) at E10.5. Markers used are indicated. **a, e**, Wild-type (arrow) and **b, g**, ectopic (arrowhead) expression of *Otx2* in the mutants in the midbrain and anterior hindbrain of rh1; ectopic expression of *Pax2* (**d, h**) compared with wild-type (**c, f**); and expansion of *En1* (**j**) expression in the mutants compared with the wild-type (**i**).

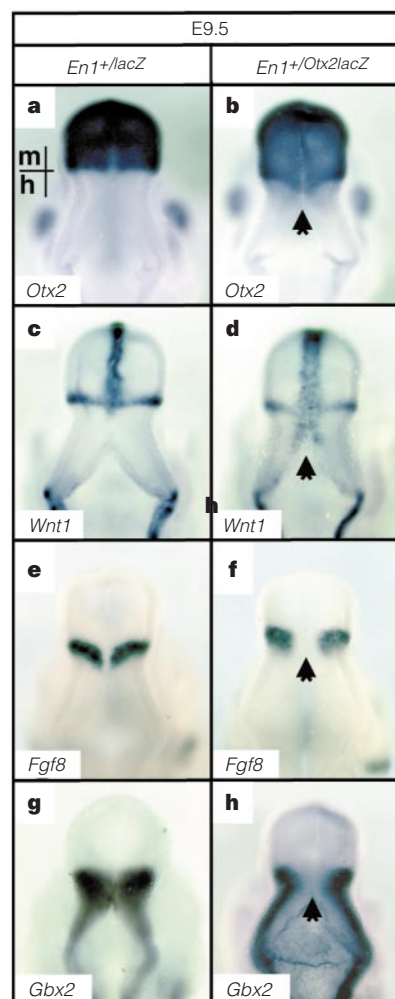


Figure 5 mRNA *in situ* hybridization of mid/hindbrain-specific markers in control *En1*^{+/lacZ} (**a, c, e, g**) and mutant *En1*^{+/Otx2lacZ} (**b, d, f, h**) embryos at E9.5. Markers used are indicated. **a**, Wild-type and **b**, ectopic expression of *Otx2* in the mutant at the level of the hindbrain of rh1 (arrow). **d**, Ectopic *Wnt1* expression is observed in the dorsal rh1 (arrow) compared with wild-type (**c**). **f**, *Fgf-8* expression is slightly shifted caudally into rh1 abutting the ectopic *Otx2* expression domain but is repressed in the dorsal midline (arrow). **h**, *Gbx2* expression is repressed in the dorsal, anterior hindbrain at the site of ectopic *Otx2* expression. h, hindbrain; m, midbrain.

zebrafish and chicken have shown that *Wnt1* and *Fgf-8* are necessary to maintain the expression of *En1*, *En2* and *Pax2*^{7,8,10,19–22}. Moreover, *En1* is necessary to maintain *Pax2* and *Wnt1* (V. Blanquet and W.W., manuscript in preparation) and *Wnt1* and *Pax2* maintain *En1* in a cross-regulatory loop^{7,23–25}. The inactivation of one of these genes disrupts the cross-regulatory genetic interactions and, subsequently, causes loss of the mid/hindbrain tissue^{10,21,22,24,25}. Studies of *Otx2*^{−/−} and *Otx1*^{+/+}, *Otx2*^{+/+} and *Otx1*^{−/−}, *Otx2*^{+/+} compound mouse mutants indicated that the proper development and patterning of the forebrain, midbrain and anterior hindbrain depends on the *Otx* gene dosage^{2,26,27} in the neural plate and/or underlying tissues. Here, we show that the formation and positioning of the isthmus organizer is determined by the caudal expression limit of *Otx2* within the neuroectoderm, which also specifies midbrain versus hindbrain development. Thus, *Otx2* expression and the regulation of its caudal limit could be placed upstream of the genetic events leading to isthmus organizer formation and localization (Fig. 6). □

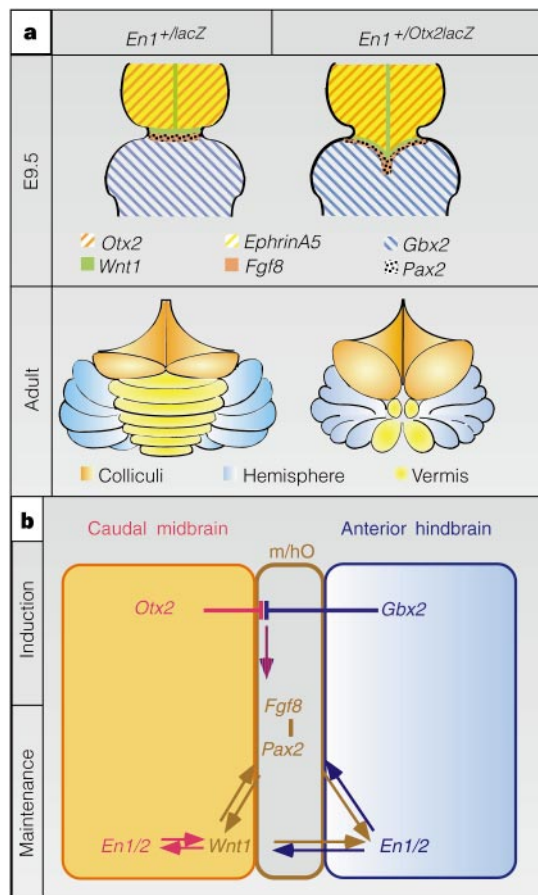


Figure 6 Genetic interactions leading to isthmus organizer (IsO) formation and mid/hindbrain specification. **a**, At E9.5, *Otx2* is expressed in the midbrain with a sharp limit of expression at the IsO and *Gbx2* is expressed in the hindbrain and IsO with a sharp boundary of expression at the midbrain border abutting *Otx2* expression. *Fgf-8* and *Pax2* are expressed in the IsO overlapping with *Gbx2*. *Wnt1* expression forms a narrow stripe in the caudal midbrain adjacent to the IsO and *Ephrin-A5* is expressed in the midbrain, both overlapping with *Otx2* expression. *En1* and *2* are expressed across the mid/hindbrain region (not shown). Ectopic expression of *Otx2* in the IsO and anterior dorsal hindbrain (right panel) leads to a shift of *Fgf-8*, *Pax2*, *Gbx2*, *Ephrin-A5* and *Wnt1* expression caudally early in embryonic development, resulting in reduction of the vermis and enlargement of the colliculi in the adult brain (bottom right). **b**, Genetic hierarchy determining IsO formation and localization based on gain-of-function and loss-of-function experiments of *En1*, *Pax2*, *Wnt1*, *Fgf-8*, *Gbx2* and *Otx2*. As shown here, ectopic expression of *Otx2* in IsO and rh1 results in a caudal shift of *Gbx2*, *Pax2*, *Fgf-8* and *Wnt1* expression. Thus, *Otx2* can be placed upstream in the hierarchy of genetic interactions specifying the IsO and midbrain versus hindbrain fate. m/hO, mid/hindbrain organizer.

Methods

Knock-in of a murine *Otx2* minigene into the *En1* locus.

The *En1* knock-in vector has been described²⁸. The *PGK-neo* cassette is flanked by *loxP* sites, allowing the removal of the neomycin gene cassette. A mouse *Otx2* complementary DNA encompassing the entire coding sequences followed by an encephalomyocarditis-virus-derived internal ribosome entry site (IRES) sequence linked to a bacterial *lacZ* reporter gene was inserted into the *KpnI* site of the *En1* knock-in vector²⁸, placing the *Otx2* minigene downstream of the *En1* promoter. R1 ES cells were cultured, electroporated and selected as described²⁴. Recombinant clones were identified by Southern blotting using *HindIII* and *XbaI* restriction enzymes. As a 5' probe, a 1.2-kb *SacI* fragment and, as a 3' probe, a 700-bp *EcoRI*–*HindIII* fragment were used to identify a 7-kb or 4.5-kb fragment after a proper homologous recombination event. A total of 300 colonies were analysed, yielding 21 recombinant clones.

Two independent targeted ES cell clones were injected into C57BL/6 blastocysts. Germline chimaeras were bred with either C57BL/6 or 129/Sv mouse strains. Germline transmission was detected by Southern blot analysis after *HindIII* digestion of genomic DNA and hybridization with a 3' probe. F1 offspring were mated with the deleter transgenic mouse line expression cre-recombinase ubiquitously¹⁶. Cre-mediated *neo* excision was monitored by three PCR reactions detecting the presence of *lacZ* and *cre* and confirming the loss of the *neo* sequence. *LacZ* primers: sense, 5'-GGTGGCGCTGGATGGAAGC-3'; antisense, 5'-CGCCATTGACCACTACC-3'. *Neo* primers: sense, 5'-CTGGGCACAACAGACAATCGG-3'; antisense, 5'-CGATAGAGGGC-GATGCGCTGC-3'. *Cre* primers: sense, 5'-GATCGCTGCCAGGATATACG; antisense, 5'-CATCGCCATCTTCAGCAG.

Histology, immunohistochemistry and *in situ* hybridization.

Age-matched embryos and tissues were dissected, fixed in 4% paraformaldehyde (PFA), dehydrated and embedded in paraffin for microtome sections. Haematoxylin–eosin was used for staining. For β -galactosidase staining the tissue was fixed in 2% PFA and 0.2% glutaraldehyde for 5–10 min and further processed as described²⁴. Immunohistochemistry was carried out on 20- μ m sections of fresh tissue or paraffin embedded as described using antisera recognizing *Otx2* (ref. 29), calbindin D-28 (Swant Swiss Antibodies) and calretinin (Swant Swiss Antibodies). *In situ* hybridizations were performed as described⁶ with antisense RNA probes transcribed from plasmids containing fragments of *Otx2* (ref. 26), *En1* (ref. 15), *En2* (ref. 15), *Fgf-8* (ref. 8), *Wnt1* (ref. 7), *Ephrin-A5* (ref. 30) and *Gbx2* (ref. 6).

Received 5 May; accepted 14 July 1999.

- Simeone, A., Acampora, D., Massino, G., Stornaiolo, A. & Boncinelli, E. Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358**, 687–690 (1992).
- Acampora, D. & Simeone, A. Understanding the roles of *Otx1* and *Otx2* in the control of brain morphogenesis. *Trends Neurosci.* **22**, 116–122 (1999).
- Lumsden, A. & Krumlauf, R. Patterning the vertebrate neuraxis. *Science* **274**, 1109–1115 (1996).
- Beddington, R. S. P. & Robertson, E. J. Anterior patterning in mouse. *Trends Genet.* **14**, 277–284 (1998).
- Ang, S.-L., Conlon, R. A., Jin, O. & Rossant, J. Positive and negative signals from mesoderm regulate the expression of mouse *Otx2* in ectoderm explants. *Development* **120**, 2979–2989 (1994).
- Wassarman, K. M. *et al.* Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function. *Development* **124**, 2923–2934 (1997).
- McMahon, A. P., Joyner, A. L., Bradley, A. & McMahon, J. A. The midbrain–hindbrain phenotype of *Wnt-1*^{−/−} mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69**, 581–595 (1992).
- Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. R. & Martin, G. R. FGF8 induces formation of an ectopic isthmus organizer and isthmocerebellar development via a repressive effect on *Otx2* expression. *Development* **126**, 1189–1200 (1999).
- Bally-Cuif, L. & Wassef, M. Determination events in the nervous system of the vertebrate embryo. *Curr. Opin. Genet. Dev.* **5**, 450–458 (1995).
- Joyner, A. L. Engrailed, *Wnt* and *Pax* genes regulate midbrain–hindbrain development. *Trends Genet.* **12**, 15–20 (1996).
- Wassef, M. & Joyner, A. L. Early mesencephalon/metencephalon patterning and development of the cerebellum. *Prospect. Dev. Neurobiol.* **5**, 3–16 (1997).
- Martinez, S., Wassef, M. & Alvarado-Mallart, R. M. Induction of amesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the homeobox gene *en*. *Neuron* **6**, 971–981 (1991).
- Martinez, S., Marin, F., Nieto, M. A. & Puellas, L. Induction of ectopic engrailed expression and fate change in avian rhombomeres: intersegmental boundaries as barriers. *Mech. Dev.* **51**, 289–303 (1995).
- Meinhardt, H. Cell determination boundaries as organizing regions for secondary embryonic fields. *Dev. Biol.* **96**, 375–385 (1983).
- Davis, C. A. & Joyner, A. L. Expression patterns of the homeobox containing genes *En1* and *En2* and the proto-oncogene *int-1* diverge during mouse development. *Gen. Dev.* **2**, 1736–1744 (1988).
- Schwenk, F., Baron, U. & Rajewsky, K. A cre-transgenic mouse strain for ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nud. Acids Res.* **24**, 5080–5081 (1995).
- Millet, S., Bloch-Gallego, E., Simeone, A. & Alvarado-Mallart, R.-M. The caudal limit of *Otx2* gene expression as a marker of the midbrain/hindbrain boundary: a study using *in situ* hybridisation and chick–quail homotopic grafts. *Development* **122**, 3785–3797 (1996).
- Marin, F. & Puellas, L. Morphological fate of rhombomeres in quail/chick chimeras: a segmental analysis of hindbrain nuclei. *Eur. J. Neurosci.* **7**, 1714–1738 (1995).
- Danielian, P. S. & McMahon, A. P. Engrailed-1 as a target of the *Wnt-1* signalling pathway in vertebrate midbrain development. *Nature* **383**, 332–334 (1996).
- Lee, S. M. K., Danielian, P. S., Fritsch, B. & McMahon, A. P. Evidence that FGF8 signalling from the midbrain–hindbrain junction regulates growth and polarity in the developing midbrain. *Development* **124**, 959–969 (1997).

21. Meyers, E. N., Lewandoski, M. & Martin, G. R. An *Fgf8* mutant allelic series generated by Cre- and Flp-mediated recombination. *Nature Genet.* **18**, 136–141 (1998).
22. Reifers, F. *et al.* *Fgf8* is mutated in zebrafish acerebellar (*ace*) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125**, 2381–2395 (1998).
23. Lun, K. & Brand, M. A series of no isthmus (*noi*) alleles of the zebrafish *pax2.1* gene reveals multiple signalling events in development of the midbrain-hindbrain boundary. *Development* **125**, 3049–3062 (1998).
24. Wurst, W., Auerbach, A. B. & Joyner, A. Multiple developmental defects in *Engrailed-1* mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120**, 2065–2075 (1994).
25. Schwarz, M., Alvarez-Bolado, G., Urbánek, P., Busslinger, M. & Gruss, P. Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: Evidence from targeted mutation. *Proc. Natl Acad. Sci. USA* **94**, 14518–14523 (1997).
26. Acampora, D., Avantaggiato, V., Tuorto, F. & Simeone, A. Genetic control of brain morphogenesis through *Otx* gene dosage requirement. *Development* **124**, 3639–3650 (1997).
27. Suda, Y., Matsuo, I. & Aizawa, S. Cooperation between *Otx1* and *Otx2* genes in developmental patterning of rostral brain. *Mech. Dev.* **69**, 125–141 (1997).
28. Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B. & Joyner, A. L. Rescue of the *En1* mutant phenotype by replacement of *En1* with *En2*. *Science* **269**, 679–682 (1995).
29. Mallamaci, A., Di Blas, E., Briata, P., Boncinelli, E. & Corte, G. OTX2 homeoprotein in the developing central nervous system and migratory cells of the olfactory area. *Mech. Dev.* **58**, 165–178 (1996).
30. Flenniken, A. M., Gale, N. W., Yancopoulos, G. D. & Wilkinson, D. G. Distinct and overlapping expression patterns of ligands for Eph-related receptor tyrosine kinases during mouse embryogenesis. *Dev. Biol.* **179**, 382–401 (1996).

Acknowledgements

We thank L. Bally-Cuif, J. Favor and M. Wassef for critically reading the manuscript; A. Nagy for R1 ES cells; G. Corte, R. Hawkes, A. Joyner, G. Martin, A. Mallamaci, A. Simeone and D. Wilkinson for antibodies and probes; K. Rajewski for the *cre*-expressing transgenic mouse line; P. Westphal, A. Drexler-Kurz and B. Klädtker for technical support; and S. Rengsberger and A. Maier for secretarial assistance and artwork. This work was supported by EU Biotech (W.W.), EU Biomed (E.B. and W.W.), HSFP (W.W.) and the Deutsche Forschungsgemeinschaft (SFP 190, W.W.).

Correspondence and requests for material should be addressed to W.W. (e-mail: Wurst@mpipsykl.mpg.de).

Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation

Setsuko Sahara^{*†}, Mamoru Aoto^{*†}, Yutaka Eguchi^{*†}, Naoko Imamoto[‡], Yoshihiro Yoneda[‡] & Yoshihide Tsujimoto^{*†}

^{*} Department of Medical Genetics, Biomedical Research Center, [‡] Department of Anatomy and Cell Biology, Osaka University Medical School, and [†] CREST of Japan Science and Technology Corporation (JST), 2-2 Yamadaoka, Suita, Osaka 565–0871, Japan

Apoptosis is defined by several unique morphological nuclear changes, such as chromatin condensation and nuclear fragmentation¹. These changes are triggered by the activation of a family of cysteine proteases called caspases^{2,3}, and caspase-activated DNase (CAD/DFF40)^{4,5} and lamin protease (caspase-6)^{6,7} have been implicated in some of these changes. CAD/DFF40 induces chromatin condensation in purified nuclei, but distinct caspase-activated factor(s) may be responsible for chromatin condensation⁸. Here we use an *in vitro* system to identify a new nuclear factor, designated Acinus, which induces apoptotic chromatin condensation after cleavage by caspase-3 without inducing DNA fragmentation. Immunodepletion experiments showed that Acinus is essential for apoptotic chromatin condensation *in vitro*, and an antisense study revealed that Acinus is also important in the induction of apoptotic chromatin condensation in cells.

To identify the molecules acting downstream of caspases that are responsible for apoptotic changes of the nucleus, we have developed an *in vitro* system using digitonin-permeabilized cells, that has been

widely used to study active nuclear transport⁹. When permeabilized human HeLa cells were incubated with a lysate prepared from apoptotic Jurkat cells, apoptotic morphological changes of the nucleus, including chromatin condensation, were induced (Fig. 1a). The lysate from live Jurkat cells treated with caspase-3 induced chromatin condensation, but the lysate or the caspase-3 alone failed to induce this (Fig. 1a) indicating that the target molecule of caspase-3 that is responsible for chromatin condensa-

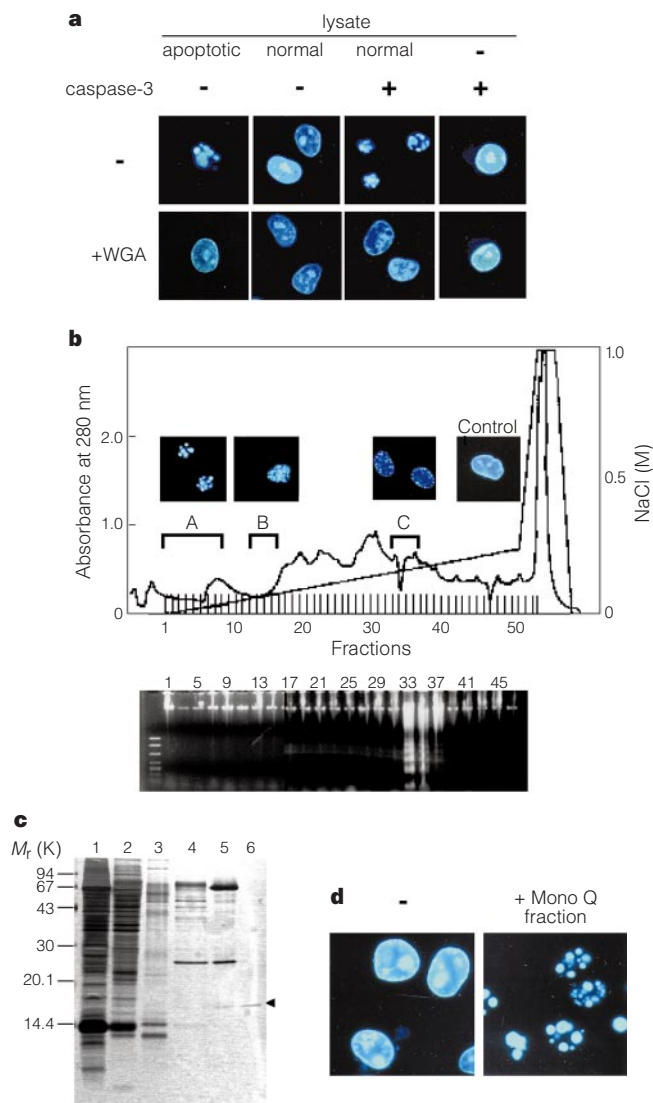


Figure 1 Purification of an apoptotic chromatin-condensation factor from bovine thymus. **a**, *In vitro* apoptosis system using permeabilized HeLa cells. Permeabilized cells were incubated for 2 h with apoptotic Jurkat lysate, normal Jurkat lysate, normal Jurkat lysate plus caspase-3, or caspase-3 only, with or without 0.1 mg ml⁻¹ wheat germ agglutinin (WGA), an inhibitor of active nuclear transport, and examined under a fluorescence microscope after Hoechst 33342 staining. **b**, Separation of three distinct activities inducing chromatin condensation by HiTrap-Q column chromatography of bovine thymus lysate. Fractions (2.5 μl) were assayed for induction of chromatin condensation using the *in vitro* system. Chromatin condensation caused by three fractions (A, B and C) are shown. The DNA laddering activity of every other fraction was also assayed²⁵. Protein concentration was monitored by absorbance at 280 nm. **c**, Portions of pooled fractions with chromatin-condensing activity at each step of purification were subjected to SDS-PAGE and silver staining. Lane 1, 100,000g supernatant (3.4 μg); lane 2, HiTrap-Q (1.7 μg); lane 3, Heparin Sepharose after passing the hydroxapatite column (150 ng); lane 4, Phenyl Sepharose (70 ng); lane 5, Superose 12 (50 ng); lane 6, Mono-Q (2.5 ng). Arrowhead, position of purified Acinus p17 protein. **d**, Nuclear changes caused by the Mono-Q fraction. Permeabilized HeLa cells were incubated with (right) or without (left) the active fraction from Mono-Q column chromatography in the absence of caspase-3.

tion was present in the lysate. Chromatin condensation was inhibited by the addition of wheatgerm agglutinin¹⁰ (Fig. 1a), indicating that active nuclear transport is involved.

We used this assay system to purify factors that induce apoptotic chromatin condensation from bovine thymus lysate. Because a fraction of the bovine thymocytes were apoptotic, we expected to find inducer(s) of apoptotic nuclear changes in the lysate as either proforms or active forms. We therefore carried out the assay in the presence of active caspase-3 to convert any proforms to their active forms. Components essential for active nuclear transport were

supplemented to help proteins enter the nucleus. Three fractions (peaks A, B and C) that induced chromatin condensation were detected after HiTrap Q column chromatography of bovine lysate. The factor in peak A caused typical apoptotic chromatin condensation without inducing oligonucleosomal DNA fragmentation (Fig. 1b). By microsequencing the purified protein (data not shown) we identified the factor in peak B as the proform of caspase-6, a protease that cleaves lamin A^{6,7}. The factor in peak C was identified as a bovine CAD/ICAD (DFF40/45) that induces oligonucleosomal DNA fragmentation after cleavage by caspase-3 (Fig. 1b).

Table 1 Purification of Acinus

Step	Protein (mg)*	Total activity (units)†	Specific activity (units mg ⁻¹)	Purification (fold)	Yield (%)
Supernatant (100,000g)	3,680	n.d.	n.d.	n.d.	n.d.
HiTrap-Q	186	14,400	77.4	1	100
Hydroxyapatite/Heparin Sepharose	2.8	4,000	1,428	18.4	27.8
Phenyl Sepharose	0.5	1,680	3,360	43.4	11.7
Superose 12	0.1	1,600	16,000	207	11.1
Mono-Q	0.001	1,120	1,120,000	14,470	7.78

n.d., not determined owing to other factors that affected nuclear morphology.

*Amounts of protein were determined using the DC protein assay kit (BIO-RAD), and those of Mono-Q pools were estimated by silver staining after SDS-PAGE.

†One unit is defined as the activity inducing nuclear chromatin condensation in 50% of cells.

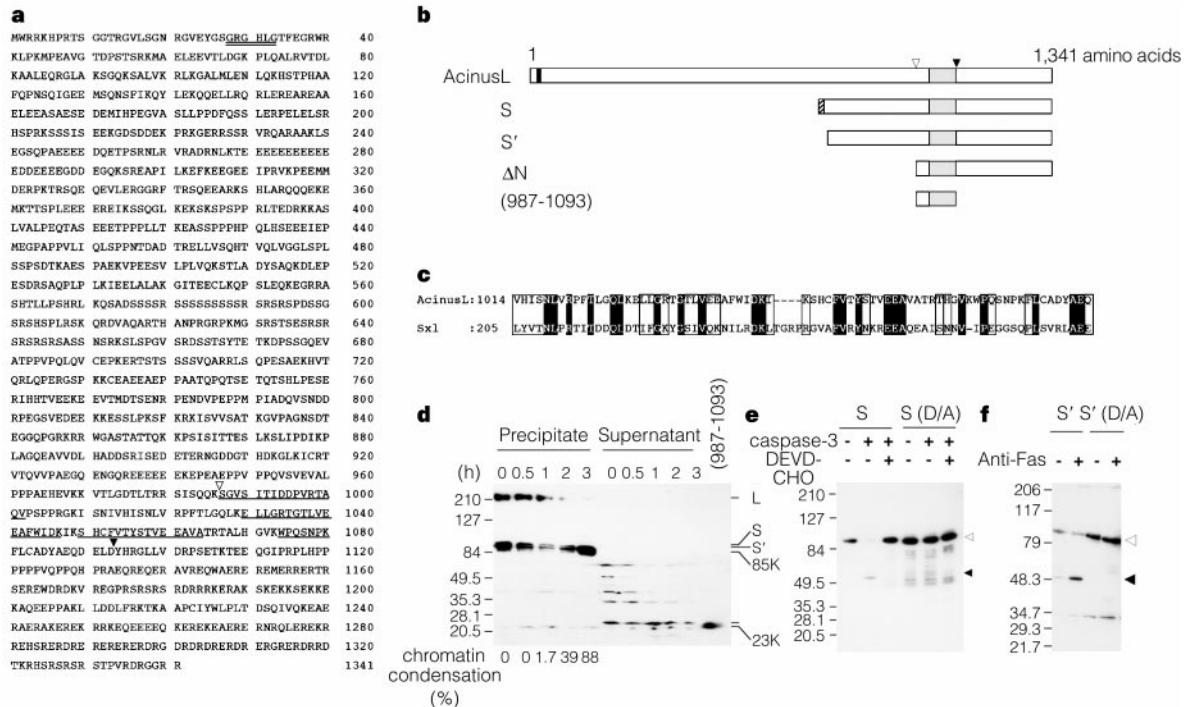


Figure 2 Primary structure of Acinus, and cleavage of Acinus by caspase-3. **a**, The predicted amino-acid sequence of human AcinusL. The sequences of four peptides obtained from purified bovine Acinus p17 are underlined. Open triangle, N terminus of Acinus p17; filled triangle, cleavage site for caspase-3; double underline, putative P-loop site. **b** AcinusS has a unique sequence (MLSESKEG, hatched box) at the N terminus followed by residues 767–1341 of AcinusL. AcinusS' corresponds to residues 774–1341 of AcinusL. **c** Acinus(987–1093) correspond to residues 987–1341 and 987–1093, respectively. Black box, P-loop; grey box, region homologous to the RNA-recognition motif of Sxl. **d**, Comparison of the amino-acid sequences of Acinus and *Drosophila* Sxl. Identical and conserved amino acids are shown by closed and open boxes, respectively. **e**, Cleavage of Acinus during Fas-mediated apoptosis in cells. Jurkat cells were treated with 0.1 $\mu\text{g ml}^{-1}$ anti-Fas antibody for the periods indicated and lysed with the lysis buffer containing 1% TritonX-100. After centrifugation, supernatant and pellet were dissolved in SDS sample buffer and subjected to SDS-PAGE, together with rAcinus(987–1093), which had been expressed in reticulocyte lysate as a His-tagged protein and then cleaved off His-tag by enterokinase. The proteins were transferred to a

membrane and analysed by using anti-Acinus antibody. Positions of acinusL, S, S' and cleaved fragments (85K and 23K) are shown. The percentage of cells showing apoptotic chromatin condensation is shown underneath. **f**, *In vitro* cleavage of acinus by caspase-3. AcinusS and S(D/A), in which Asp 1093 was substituted by Ala, were transiently expressed in COS-7 cells. Cell lysates in lysis buffer that contained a significant amount of AcinusS, probably because they were produced in a large quantity, were incubated for 1 h with or without caspase-3 (2.5 ng) in the presence or absence of 10 μM DEVD-CHO, and subjected to SDS-PAGE followed by immunoblotting with anti-Acinus antibody. Full-length AcinusS and the caspase-3 cleavage products are marked by open and filled arrowheads, respectively. **g**, Cleavage of flag-tagged AcinusS' in HeLa cells treated with anti-Fas antibody. HeLa cells transfected with DNA for flag-tagged AcinusS' or S'(D/A) were incubated with 1 $\mu\text{g ml}^{-1}$ anti-Fas antibody for 3 h, and cell lysates were subjected to immunoblot analysis using anti-flag antibody. Full-length AcinusS' and the cleavage product are marked by open and closed arrowheads, respectively. The same blot was also probed with anti-caspase-3 antibody, revealing a similar amount of cleavage of caspase-3 in both Fas-treated samples (data not shown).

From the peak A fractions, we used a seven-step procedure to purify a protein with a relative molecular mass of $\sim 17,000$ (M_r 17K) (Fig. 1c and Table 1). This p17 protein induced apoptotic chromatin condensation in the absence of caspase-3 (Fig. 1d), suggesting that it was an active form. We named the gene for this factor *acinus* (for apoptotic chromatin condensation inducer in the nucleus, meaning 'grape' or 'berry' in Latin) because of the 'grape-like' appearance of condensed chromatin.

Four internal peptide sequences from p17 corresponded to the predicted amino-acid sequences of a human KIAA clone (KIAA0670)¹¹ in the database (underlined in Fig. 2a). Thus, KIAA0670 seemed to represent a human homologue of *acinus*. By using the KIAA clone, we obtained three isoforms of human *acinus* complementary DNA (the L, S and S' forms), which were probably generated by alternative splicing. The *acinus*L, S, and S' cDNA contained open reading frames of 1,341, 583 and 568 amino-acid residues, respectively (Fig. 2a, b). Mouse *acinus* cDNA was also obtained, and the mouse AcinusS and S' showed 94.5 and 95.2% homology of their amino-acid sequences with the human AcinusS and S', respectively (analysis of mouse AcinusL was not completed). A search of the BLAST protein database revealed that Acinus contained a region similar to the RNA-recognition motif of *Drosophila* Sxl¹² (Fig. 2c). Only AcinusL had a P-loop motif¹³ for nucleotide binding near the amino terminus (Fig. 2a, b).

An anti-Acinus antibody was raised that recognized AcinusL, S and S' expressed from their respective cDNAs in COS-7 cells: their apparent M_s on SDS-polyacrylamide gels were 220K, 98K and 94K, respectively (data not shown). The antibody revealed that AcinusL, S, and S' along with small fragments were present in Jurkat cells (Fig. 2d). The majority of three forms of *acinus* was recovered in the fraction insoluble in TritonX-100. Most of the small fragments in the soluble fraction might have been proteolytically processed (Fig. 2d). During Fas-mediated apoptosis, AcinusL, S and S', as well as some of the small fragments, were reduced before chromatin condensation was apparent. An 85K protein in the insoluble fraction and a 23K protein in the soluble fraction appeared at the same time as chromatin condensation (Fig. 2d). These data indicate that Acinus may have been cleaved by caspase(s), generating active fragments. AcinusS was cleaved by caspase-3 *in vitro* (Fig. 2e) at Asp 1093 of DELD 1093, as determined by microsequencing the cleavage product of recombinant AcinusS. This finding is consistent with caspase-3 having a consensus DxxD target sequence. A mutation that replaced Asp1093 with Ala (D/A) abolished both the *in vitro* cleavage of AcinusS by caspase-3 (Fig. 2e) and the apoptosis-associated cleavage of AcinusS' during Fas-mediated apoptosis (Fig. 2f).

We then attempted to identify an active form of human Acinus that induced apoptotic chromatin condensation in our *in vitro* system. Purified active bovine Acinus p17 seemed to be truncated at both N- and C-terminal sites, as N-terminal sequence analysis revealed that p17 started at Ser987 (data not shown; see Fig. 2a) and the size of p17 indicated C-terminal truncation. We therefore prepared several forms of recombinant Acinus (rAcinus): AcinusS, ΔN (from Ser 987 to the C terminus); $\Delta N(D/A)$; and Acinus(987–1093), from Ser 987 to Asp 1093 (Fig. 2b). rAcinus ΔN condensed chromosomes in the presence of caspase-3, but not in the presence of the caspase-3 inhibitor DEVD-CHO or in the absence of caspase-3, whereas rAcinus $\Delta N(D/A)$, with the Asp/Ala mutation, did not induce chromatin condensation in the presence of caspase-3 (Fig. 3a). These results indicate that cleavage of rAcinus ΔN by caspase-3 at Asp 1093 was essential for chromatin condensation. rAcinus ΔN that was preincubated with caspase-3 induced chromatin condensation even in the presence of DEVD-CHO (Fig. 3a), indicating that caspase-3 activity was not required for chromatin condensation once Acinus had been cleaved by caspase-3. In support of this idea, rAcinus(987–1093) induced chromatin condensation in the absence of caspase-3 (Fig. 3a), as did bovine Acinus

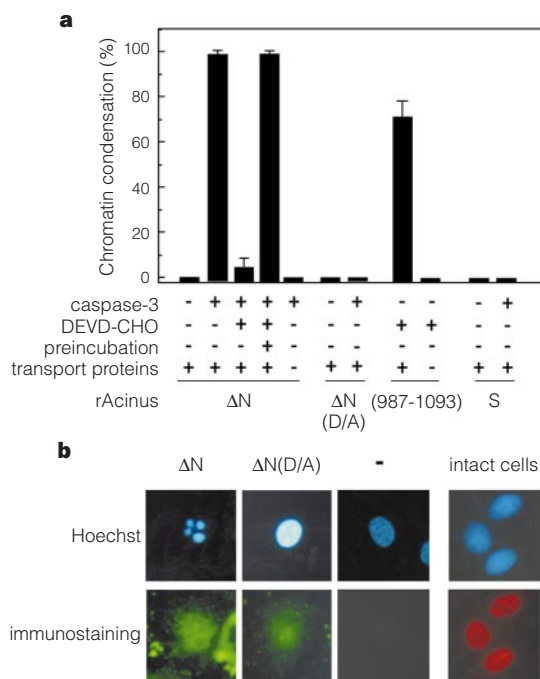


Figure 3 Induction of chromatin condensation in permeabilized cell nuclei by recombinant Acinus, and nuclear localization of Acinus. **a**, Induction of chromatin condensation by rAcinus depends on caspase-3 and nuclear transport. rAcinus ΔN (0.05 μ g), rAcinus $\Delta N(D/A)$ (0.06 μ g), rAcinus(987–1093) (0.1 μ g) and rAcinusS (0.1 μ g) were added to permeabilized HeLa cells in the presence or absence of caspase-3, DEVD-CHO and nuclear transport proteins, and incubated for 2 h. Preincubation of rAcinus ΔN with caspase-3 was at 37 °C for 1 h. Chromatin condensation was assessed under a fluorescence microscope after staining with Hoechst 33324. Data are the mean $\pm$ s.d. ($n = 4$). **b**, Nuclear localization of Acinus in permeabilized and intact cells. Permeabilized HeLa cells were incubated with His-tagged rAcinus ΔN and $\Delta N(D/A)$ followed by immunostaining with anti-Xpress antibody (Invitrogen) and FITC-conjugated secondary antibody²⁶. Intact HeLa cells were immunostained with anti-Acinus antibody and RITC-conjugated secondary antibody. Nuclei were also visualized by Hoechst 33342 staining.

p17 (Fig. 1d). Full-length rAcinusS did not condense chromatin (Fig. 3a), suggesting that either cleavage at Ser 987 or N-terminal truncation was necessary, although it remains possible that rAcinusS was not properly folded. Together with the observation that apoptotic Jurkat cells contained a new 23K protein (the same size as rAcinus(987–1093)) (Fig. 2d), these data indicate that Acinus(987–1093) was one of the active forms of Acinus to induce chromatin condensation. Acinus undergoes several proteolytic cleavages during apoptosis, so identification of other intracellular active forms must await further investigation.

Active nuclear-transport proteins were also required for the induction of chromatin condensation by rAcinus (Fig. 3a), indicating that nuclear localization of Acinus was essential for chromatin condensation in our *in vitro* system. A large amount of rAcinus ΔN translocated to the nucleus *in vitro*, as shown by immunostaining of permeabilized cells (Fig. 3b). The nuclear localization of rAcinus $\Delta N(D/A)$ indicated that cleavage of Acinus by caspase-3 was not necessary for nuclear import (Fig. 3b). Endogenous Acinus was detected mainly in the nucleus (Fig. 3b), suggesting that caspase(s) might be transported into the nucleus where proteolytically activates Acinus during apoptosis. Alternatively, a small amount of Acinus may be cleaved by caspases in the cytoplasm before being transported to the nucleus.

Consistent with the observation that bovine Acinus p17 caused chromatin condensation without oligonucleosomal DNA fragmentation (Fig. 1b), no oligonucleosomal and larger-scale fragmentation of DNA was observed when rAcinus induced chromatin

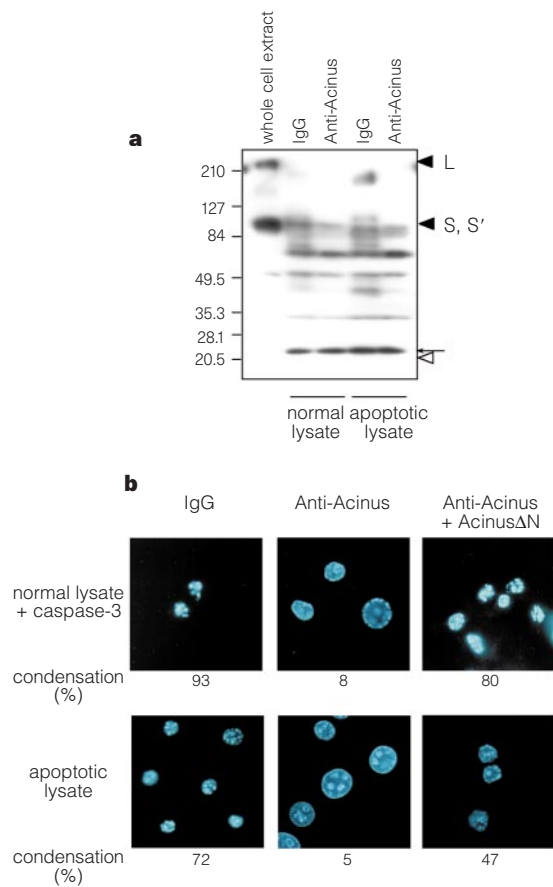


Figure 4 Role of Acinus for chromatin condensation *in vitro*. **a**, Immunodepletion of Acinus from Jurkat lysates. Lysates of untreated Jurkat cells (normal) and Jurkat cells treated for 3 h with anti-Fas antibody (apoptotic) were incubated with control IgG- or anti-Acinus antibody-conjugated protein G Sepharose. Treated lysates were resolved by SDS-PAGE and immunoblotted with anti-Acinus antibody. A whole-cell extract of normal Jurkat cells in SDS sample buffer was loaded on the left lane to assess endogenous Acinus. Arrowheads, positions of AcinusL, S, S' and Acinus(987–1093). Arrow, an unidentified protein different from Acinus(987–1093). **b**, Failure of Acinus-immunodepleted lysate to induce chromatin condensation and restoration of activity by rAcinusΔN. Normal lysate (5 μl) immunodepleted using antibodies was added with or without rAcinusΔN (0.5 μg) to permeabilized cells in the presence of caspase-3. The immunodepleted lysate from anti-Fas antibody-treated cells (apoptotic lysate) was added in the absence of caspase-3. Nuclei were visualized by Hoechst 33342 staining. The percentage of nuclei showing chromatin condensation is indicated below each fluorescence micrograph.

condensation *in vitro* (data not shown).

To determine whether Acinus was essential for apoptotic chromatin condensation, we tested the ability of normal and apoptotic Jurkat cell lysates immunodepleted of Acinus to induce chromatin condensation *in vitro*. A reduction in the amount of some forms of Acinus and its cleaved products in the normal and apoptotic lysates was confirmed by immunoblotting (Fig. 4a), whereas CAD/DFF40 activity, as assessed with the permeabilized cells, remained unchanged (data not shown). Lysates immunodepleted using the anti-Acinus antibody but not a control antibody failed to induce chromatin condensation (Fig. 4b). Addition of rAcinusΔN restored the chromatin-condensing ability of the immunodepleted lysates (Fig. 4b). These results indicated that Acinus is required for apoptotic chromatin condensation in our *in vitro* system.

We examined the role of acinus in apoptosis by transient transfection of sense and antisense *acinusL* cDNA into HeLa cells. The sense plasmid caused a slight induction of chromatin condensation without any apoptotic stimuli, and it accelerated etoposide-

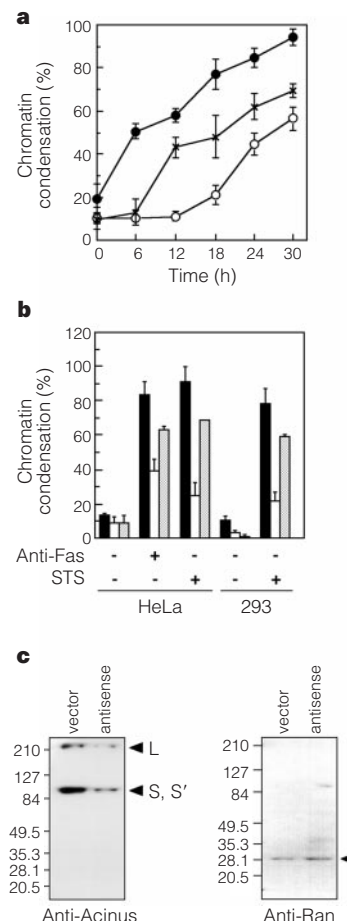


Figure 5 Role of Acinus for apoptotic chromatin condensation in cells. **a**, **b**, Increased and reduced chromatin condensation in cells transfected with *acinusL* plasmids in the sense and anti-sense orientation, respectively. HeLa (**a**, **b**) and 293 (**b**) cells transfected with 1 μg pCAGGS-*acinusL* (sense, filled circle, filled bar), pCAGGS-*acinusL*(R) (antisense, open circle, open bar) or vector (crosses, hatched bar), together with a plasmid expressing green fluorescent protein (GFP) (0.1 μg). After 48 h, cells were further incubated in the presence of 100 μM etoposide (**a**), for 2 h with 1 μg ml⁻¹ anti-Fas antibody or for 9 h with 1 μM staurosporine (STS) (**b**). Chromatin condensation in GFP-positive cells was assessed by Hoechst 33342 staining. Results are mean ± s.d. (*n* = 4 in **a**, *n* = 3 in **b**). **c**, Reduction of endogenous Acinus by transfection with the *acinusL*(R) antisense construct. HeLa cells (0.5 × 10⁶) were transfected with 1 μg pCAGGS-*acinusL*(R) plasmid or vector. After 48 h, whole-cell extracts were made in SDS sample buffer, and subjected to SDS-PAGE followed by immunoblotting using anti-Acinus antibody (left) and anti-Ran antibody (right). Positions of AcinusL, S, S' and Ran are indicated by arrowheads.

side-induced chromatin condensation (Fig. 5a), whereas introducing the antisense plasmid effectively delayed chromatin condensation (Fig. 5a). Downregulation of endogenous Acinus, but not the irrelevant protein Ran, after transfection with the antisense *acinus* plasmid was verified by immunoblotting (Fig. 5c). We obtained similar results using different apoptotic stimuli, including anti-Fas antibody and staurosporine, and a different cell line, 293 (Fig. 5b), indicating once again that Acinus is essential for apoptotic chromatin condensation in cells.

Our findings indicate that there are at least three distinct pathways downstream of the effector caspase-3 involved in apoptotic changes of the nucleus: (1) caspase-6, which is known to be a lamin protease and disrupts the interior structure of the nucleus^{6,7}; (2) CAD/DFF40, which causes oligonucleosomal DNA cleavage^{4,5}; and (3) Acinus, which induces chromatin condensation without DNA fragmentation. Furthermore, it has recently been shown that apoptosis-inducing factor (AIF) can induce peripheral chromatin condensation in a caspase-independent manner¹⁴. These factors

contribute to nuclear changes in apoptotic cells by acting on different targets.

In thymocytes and splenocytes of DFF45 knockout mice that also lack functional CAD/DFF40, apoptotic chromatin condensation is severely but not completely impaired¹⁵. CAD is not ubiquitously expressed in human tissues and cell lines in which apoptotic chromatin condensation is readily observed¹⁶. Because Acinus is ubiquitously expressed (data not shown), Acinus seems likely to play an important role in apoptotic chromatin condensation in general, although CAD/DFF40 might be dominant in inducing chromatin condensation in certain cell types.

Acinus is therefore essential for apoptotic chromatin condensation, and it might also be involved in nuclear structural changes occurring in normal cells. It has a P-loop motif¹³ and a region similar to the RNA-recognition motif of Sxl¹², indicating that Acinus might have ATPase and DNA/RNA-binding activity. Further studies using Acinus may improve our understanding not only of the process of apoptotic nuclear changes, but also nuclear function in viable cells. □

Methods

Reagents and materials

Two human cell lines, HeLa (D98/AH2) and Jurkat, were grown in RPMI 1640 medium containing 10% fetal bovine serum. Bovine thymus glands were obtained from a local slaughterhouse. Recombinant forms of human active caspase-3, p10, Ran, importin- α , and importin- β were purified and dialysed against transport buffer^{17–20}. A polyclonal rabbit antibody against human Acinus was raised against a synthetic peptide corresponding to amino-acid residues 987–1000 of AcinusL.

Preparation of cell lysate

Jurkat cells (4×10^6) treated with $0.1 \mu\text{g ml}^{-1}$ anti-Fas antibody CH-11 (MBL, Japan) for 3 h or untreated cells were sonicated in 0.5 ml lysis buffer⁹ and centrifuged. The supernatant was concentrated with an ultrafree-MC5,000 NMWL filter unit (Millipore) to 20–30 mg protein per ml.

In vitro apoptosis assay with permeabilized cells

Permeabilized HeLa and Jurkat cells were prepared essentially as described²¹. The standard assay mixture (5 μl) consisted of an ATP-regeneration system, 1 mM GTP, 50 ng ml^{-1} of recombinant caspase-3, 0.5 mg ml^{-1} importin- α , 0.5 mg ml^{-1} importin- β , 0.1 mg ml^{-1} Ran, 10 ng ml^{-1} p10 and the indicated column fractions. In the case of unfractionated lysate (4.5 μl), only the ATP-regeneration system was added. The reaction was started by adding the mixture to permeabilized HeLa cells on plates or to 1 μl of permeabilized Jurkat cells (1×10^6 cells), followed by incubation at 37 °C for 2 h. Nuclear changes were assessed under the fluorescent microscope after staining 10 μM Hoechst 33342.

Purification of Acinus

All procedures were carried out at 0–4 °C. Bovine thymus was homogenized in two volumes of the lysis buffer. After centrifugation, the supernatant was dialysed against buffer containing 20 mM Tris-HCl, pH 8.8, 2 mM MgCl_2 , 2 mM DTT, and 0.5 mM EGTA, and was applied to a HiTrap Q column (5 ml $\times$ 20) equilibrated with buffer A (20 mM Tris-HCl, pH 8.5, 2 mM MgCl_2 , 2 mM DTT, 0.5 mM EGTA, 2 mM β -glycerophosphate and 250 mM sucrose). Proteins were eluted with 400 ml of a linear NaCl gradient (0–0.2 M), and 8-ml fractions were collected. The active fractions were passed through a hydroxyapatite column (5 $\times$ 10 cm) and were applied to a Heparin Sepharose column (1.5 $\times$ 3 cm). The column was washed with 50 ml of buffer B (as buffer A but at pH 7.5) and bound proteins were eluted with 10 ml of buffer B containing 1 M ammonium sulphate. The active fraction was applied to a Phenyl Sepharose column (0.8 $\times$ 2 cm), and the flow-through fraction (10 ml) was supplemented with bovine serum albumin (20 $\mu\text{g ml}^{-1}$) and concentrated to 0.5 ml. The sample was then loaded onto a Superose 12 column in buffer B. Fractions with activity were diluted tenfold with buffer A and applied to a Mono-Q column. Proteins were eluted with 30 ml of a linear NaCl gradient (0–50 mM), and 1-ml fractions were collected and stored at –80 °C.

Sequence analysis

Bovine Acinus p17 from the Mono-Q fraction was electrotransferred on Immobilon membrane (Millipore) and digested with *Acromobacter* protease I²². The resulting peptides were purified and their sequences were determined by an Applied Biosystems protein sequencer (model 470A).

cDNA cloning

KIAA0670 DNA was a gift from O. Ohara¹¹. The sequence corresponding to the 5' end of *acinusL* was obtained using the RACE protocol. *acinusS'* cDNA was isolated by screening a cDNA library. *acinusS* cDNA was obtained by the polymerase chain reactions with reverse transcription as an alternatively spliced isoform with an insertion upstream of the start codon of *acinusS'*. Mouse *acinus* cDNAs were obtained by screening a library with human *acinus* cDNA.

Recombinant proteins

Full-length rAcinusS was produced as a glutathione S-transferase fusion protein, rAcinus ΔN and $\Delta\text{N}(\text{D/A})$ were as a six-histidine-tagged protein, and all proteins were purified according to the manufacturer's protocols. rAcinus(987–1093) was prepared by cleaving rAcinus ΔN with caspase-3 and was purified by HiTrap-Q column chromatography. The purity of GST–rAcinusS was estimated to be about 90% by CBB staining. His-tagged proteins were 10% pure (as assessed by immunoblotting).

Immunodepletion of Acinus

Antibody-loaded protein G-beads were prepared²³. For immunodepletion of acinus, lysates were incubated with an equal volume of the packed beads at 4 °C for 2 h with rotation. Supernatants were recovered by two rounds of brief spinning.

Transfection and analysis of nuclear changes

acinusL, *S*, *S'* and *S'(D/A)* cDNAs were cloned in the pCAGGS vector²⁴. *acinusL* cDNA was also cloned in the vector with an opposite orientation (*acinusL(R)*). cDNAs (1 μg) were transfected into 0.5×10^6 HeLa, 293 or COS cells with or without 0.1 μg pEGFP-C1 (Clontech) using Lipofectamine (Gibco). Cells were incubated for 48 h in the medium. For immunoblotting, cells were lysed with SDS sample buffer and subjected to SDS–PAGE. Cell death was induced by adding 100 μM etoposide, 1 $\mu\text{g ml}^{-1}$ of anti-Fas antibody or 1 μM staurosporine. After cells were stained with Hoechst 33342, the apoptotic cells and live cells positive for green fluorescent protein were counted.

Received 3 February; accepted 12 July 1999.

- Kerr, J. F. R., Wyllie, A. H. & Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239–257 (1972).
- Salvesen, G. S. & Dixit, V. M. Caspases: intracellular signaling by proteolysis. *Cell* **91**, 443–446 (1997).
- Thornberry, N. A. & Lazebnik, Y. Caspases: Enemies within. *Science* **281**, 1312–1316 (1998).
- Enari, M. *et al.* A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**, 43–50 (1998).
- Liu, X. *et al.* The 40-kDa subunit DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. *Proc. Natl Acad. Sci. USA* **95**, 8461–8466 (1998).
- Takahashi, A. *et al.* Cleavage of lamin A by Mch2 α but not CPP32: multiple ICE-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc. Natl Acad. Sci. USA* **93**, 8395–8400 (1996).
- Orth, K. *et al.* The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J. Biol. Chem.* **271**, 16443–16446 (1996).
- Samejima, K. *et al.* Transition from caspase-dependent to caspase-independent mechanisms at the onset of apoptotic execution. *J. Cell Biol.* **143**, 225–239 (1998).
- Adam, S. A., Marr, R. S. & Gerace, L. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* **111**, 807–816 (1990).
- Yoneda, Y., Imamoto-Sonobe, N., Yamaizumi, M. & Uchida, T. Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. *Exp. Cell Res.* **173**, 586–595 (1987).
- Ishikawa, K. *et al.* Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins *in vitro*. *DNA Res.* **5**, 169–176 (1998).
- Samuels, M. E., Schedl, P. & Cline, T. W. The complex set of late transcripts from the *Drosophila* sex determination gene *sex-lethal* encodes multiple related polypeptides. *Mol. Cell. Biol.* **11**, 3584–3602 (1991).
- Saraste, M., Sibbald, P. R. & Wittinghofer, A. The P-loop—a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* **15**, 430–434 (1990).
- Susin, S. A. *et al.* Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441–446 (1999).
- Zhang, J. *et al.* Resistance to DNA fragmentation and chromatin condensation in mice lacking the DNA fragmentation factor 45. *Proc. Natl Acad. Sci. USA* **95**, 12480–12485 (1998).
- Mukae, N. *et al.* Molecular cloning and characterization of human caspase-activated DNase. *Proc. Natl Acad. Sci. USA* **95**, 9123–9128 (1998).
- Yasuhara, N. *et al.* Essential role of active nuclear transport in apoptosis. *Genes Cells* **2**, 55–64 (1997).
- Imamoto, N. *et al.* *In vivo* evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. *EMBO J.* **14**, 3617–3626 (1995).
- Tachibana, T., Hieda, M., Sekimoto, T. & Yoneda, Y. Exogenously injected-nuclear import factor p10/NTF2 inhibits signal-mediated nuclear import and export of proteins in living cells. *FEBS Lett.* **397**, 177–182 (1996).
- Melchior, F., Sweet, D. J. & Gerace, L. Analysis of Ran/TC4 function in nuclear protein import. *Methods Enzymol.* **257**, 279–291 (1995).
- Gorlich, D., Prehn, S., Laskey, R. A. & Hartman, E. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell* **79**, 767–778 (1994).
- Matsuda, P. A. *Practical Guide to Protein and Peptide Purification for Microsequencing* (Academic, San Diego, 1993).
- Hirano, T., Kobayashi, R. & Hirano, M. Condensins, chromosome condensation protein complex containing XCAP-C, XCAP-E and a *Xenopus* homolog of the *Drosophila* Barren protein. *Cell* **89**, 511–521 (1997).
- Niwa, H., Yamamura, K. & Miyazaki, J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199 (1991).
- Enari, M., Hase, A. & Nagata, S. Apoptosis by a cytosolic extract from Fas-activated cells. *EMBO J.* **14**, 5201–5208 (1995).
- Iwahashi, H. *et al.* Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy. *Nature* **390**, 413–417 (1997).

Acknowledgements

We thank O. Ohara and J. Miyazaki for KIAA0670 plasmid and pCAGGS plasmid,

respectively, and Y. Sakamoto for help with microsequence analysis. This work was supported in part by grants for Scientific Research on Priority Areas, for COE Research, and for JSPS fellows from the Ministry of Education, Science, Sports and Culture of Japan.

Correspondence and requests for materials should be addressed to Y.T. (e-mail: tsujimoto@gene.med.osaka-u.ac.jp). The nucleotide sequence data will appear in the Genbank nucleotide sequence database under the accession numbers AF124726, AF124727, AF124728, AF124725, AF124729 for human *acinusL*, S, S' and mouse *acinus* and S', respectively.

Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP

Kam Yeung^{*†}, Thomas Seitz^{†‡}, Shengfeng Li[§],
Petra Janosch^{||}, Brian McFerran^{||}, Christian Kaiser[‡],
Frances Fee^{||}, Kostas D. Katsanakis^{||}, David W. Rose[¶],
Harald Mischak[#], John M. Sedivy^{*†} & Walter Kolch[†]

^{*} Brown University, Department of Molecular Biology, Cell Biology and Biochemistry, Richmond 02912, USA

[‡] GSF-Research Centre for Health and Environment, Institute for Clinical Molecular Biology, Marchioninstrasse 25, 81377 München, Germany

^{||} Beatson Institute for Cancer Research, CRC Beatson Laboratories, Switchback Road, Bearsden, Glasgow G61 1BD, UK

[§] University of California San Diego, Department of Medicine and Whittier Diabetes Program, 9500 Gilman Drive, La Jolla, California, 92093-0673, USA

[#] Franz-Volhard Klinikum, Max Delbrück Centre, Wiltbergstrasse 50, 13122 Berlin, Germany

[†] These authors contributed equally to this work

Raf-1 phosphorylates and activates MEK-1, a kinase that activates the extracellular signal regulated kinases (ERK). This kinase cascade controls the proliferation and differentiation of different cell types^{1,2}. Here we describe a Raf-1-interacting protein, isolated using a yeast two-hybrid screen. This protein inhibits the phosphorylation and activation of MEK by Raf-1 and is designated RKIP (Raf kinase inhibitor protein). *In vitro*, RKIP binds to Raf-1, MEK and ERK, but not to Ras. RKIP co-immunoprecipitates with Raf-1 and MEK from cell lysates and colocalizes with Raf-1 when examined by confocal microscopy. RKIP is not a substrate for Raf-1 or MEK, but competitively disrupts the interaction between these kinases. RKIP overexpression interferes with the activation of MEK and ERK, induction of AP-1-dependent reporter genes and transformation elicited by an oncogenically activated Raf-1 kinase. Downregulation of endogenous RKIP by expression of antisense RNA or antibody microinjection induces the activation of MEK-, ERK- and AP-1-dependent transcription. RKIP represents a new class of protein-kinase-inhibitor protein that regulates the activity of the Raf/MEK/ERK module.

In metazoans the Ras/Raf-1/MEK/ERK module is a ubiquitously expressed signalling pathway that conveys mitogenic and differentiation signals from the cell membrane to the nucleus¹. This kinase cascade appears to be spatially organized in a signalling complex that is nucleated by Ras proteins³. The regulation of the Ras/Raf-1/MEK/ERK module is complex and may include associations with scaffolding and regulatory proteins⁴. To isolate such proteins we used the Raf-1 kinase domain, BXB⁵, as bait in a yeast two-hybrid screen⁶. Screening 500,000 clones of a human T-cell library yielded nine clones that specifically interacted with BXB. Five clones corresponded to 14-3-3 proteins. One clone, RKIP, bound to both kinase-active and kinase-negative BXB, but not to control baits (Fig. 1a). Partial sequencing of the RKIP complementary DNA predicted a protein identical to the phosphatidylethanolamine

Prey Bait	RKIP (His- prototrophy)	RKIP (β-gal activity)
BXB	yes	yes
Kinase-dead BXB	yes	yes
cdk2 kinase	no	no
p53	no	no
Lamin	no	no

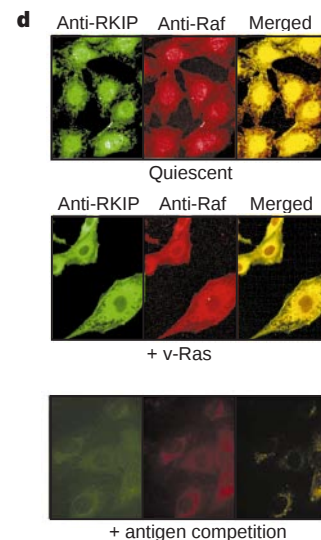
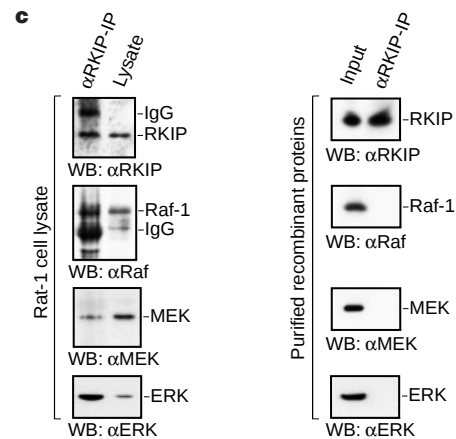
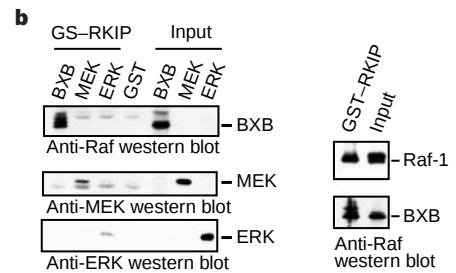


Figure 1 *In vitro* interaction of RKIP with components of the ERK pathway. **a**, RKIP interacts with BXB but not with control baits in the yeast two-hybrid system. **b**, Binding of recombinant BXB, full-length Raf-1, MEK-1 and ERK-2 to GST-RKIP beads. Input: 1% of the respective proteins used in binding reactions; GST: GST-beads. **c**, Co-immunoprecipitation of Raf-1, MEK and ERK with RKIP in Rat-1 cells. The RKIP antiserum does not precipitate recombinant Raf-1, MEK-1 and ERK-2 proteins individually. **d**, Colocalization of Raf-1 and RKIP in 208F fibroblasts by confocal microscopy. Antigen competition: antisera were pre-absorbed with their cognate antigens.

§ Present address: Cor Therapeutics Inc., South San Francisco, California 94080, USA.

binding proteins (PEBP) with relative molecular mass 23,000 ($M_r = 23K$) from humans and monkeys. These proteins are widely expressed and evolutionarily conserved, but their functions remain obscure⁷.

Purified recombinant RKIP was tested for binding to the components of the Ras/Raf-1/MEK/ERK cascade. RKIP associated with BXB, full-length Raf-1, MEK-1 and (more weakly) with ERK-2, but not with Ras. RKIP binding was independent of Raf-1 kinase activity, not affected by phosphatidylethanolamine, and direct, as shown by the interaction of purified proteins produced in *Escherichia coli* (Fig. 1b and data not shown).

These interactions were also shown between endogenous mammalian proteins. An RKIP antiserum co-immunoprecipitated Raf-1, MEK and ERK from Rat-1 cells. This was not due to cross-reactivity, because the RKIP antiserum failed to immunoprecipitate purified Raf-1, MEK-1 or ERK-2 individually (Fig. 1c). These interactions were also observed in reciprocal immunoprecipitations with antisera to Raf-1, MEK or ERK (data not shown). Confocal microscopy revealed extensive colocalization between Raf-1 and RKIP, in both quiescent and Ras-transformed cells (Fig. 1d).

To investigate the relevance of the interaction between RKIP and the kinases of the Raf/MEK/ERK module in mammalian cells, we inhibited endogenous RKIP by antibody microinjection or expression of antisense RNA. As the AP-1 transcription factor is a major target of Raf signalling⁸⁻¹⁰, we tested the influence of RKIP on AP-1 activity (Fig. 2a). Microinjection of affinity-purified anti-RKIP antibodies robustly activated a co-injected AP-1-dependent reporter gene in serum-deprived Rat-1 fibroblasts (Fig. 2a). This effect was highly specific, because the injection of control immunoglobulin (IgG) was ineffective, anti-RKIP IgG did not affect the expression of a cAMP-dependent reporter gene, and co-injection of an RKIP expression vector abolished AP-1 induction by anti-RKIP

IgG. We also downregulated RKIP expression using an RKIP antisense vector, pAS-C143. This vector markedly reduced RKIP levels without affecting the expression of MEK-1 or actin (Fig. 2b). pAS-C143 substantially induced the AP-1 reporter gene in serum-starved NIH 3T3 cells (Fig. 2c). These data confirm the microinjection results and show that RKIP suppresses the Raf/MEK/ERK pathway.

Overexpression experiments further confirmed this conclusion. RKIP transfection diminished basal as well as BXB-induced AP-1 activity (Fig. 3a), and microinjection of an RKIP expression vector impaired AP-1 induction by BXB (Fig. 3b). Notably, RKIP did not interfere with AP-1 stimulation by ERK-1. Next, we tested the effects of RKIP overexpression in transformation assays. In contrast to transient reporter-gene assays, transformation assays accommodate the complexity of cellular responses to the chronic deregulation of a single signalling component. RKIP significantly reduced the transformation efficiency of BXB in three distinct assays: morphological transformation, focus formation and anchorage-independent growth (Fig. 3c). RKIP also decreased the total colony yield, albeit to a lesser extent than transformation, showing that RKIP interferes with Raf-mediated proliferation as well as transformation. In contrast, RKIP impaired the induction of foci by *v-fos* or mutationally activated MEK alleles only to a small extent, and failed to inhibit *v-src* transformation (Fig. 3d), indicating that RKIP may specifically block transformation by the Raf/MEK/ERK pathway, primarily by inhibiting Raf.

To investigate the effects of RKIP on individual activation steps, we reconstructed the Raf/MEK/ERK cascade *in vitro* using recombinant proteins (Fig. 4a). RKIP decreased the phosphorylation of MEK by Raf-1, but did not inhibit ERK phosphorylation by MEK or ELK phosphorylation by ERK. In addition, RKIP (1) failed to inhibit MEK-DD, a constitutively active mutant of MEK¹¹, or MEK activated by TPA treatment of cells (Fig. 4b); (2) did not

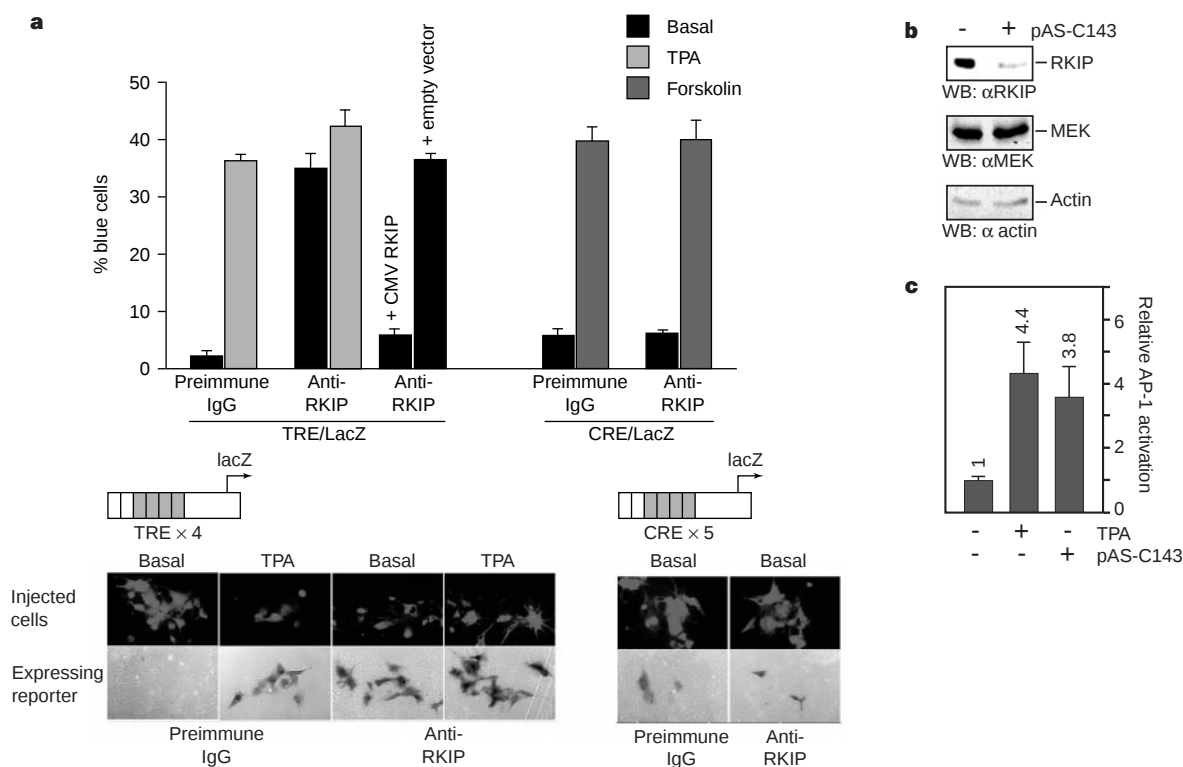


Figure 2 Inhibition of endogenous RKIP activates AP-1-dependent transcription. **a**, Microinjection of anti-RKIP antibodies. Quiescent Rat-1 cells were microinjected with the indicated reporter plasmids and antibodies and either left unstimulated or treated with 200 ng ml⁻¹ TPA or 20 µg ml⁻¹ forskolin. **b**, The RKIP antisense vector, pAS-C143, downregulates expression of endogenous RKIP. NIH 3T3 cells were cotransfected with

pAS-C143 and a GFP-expressing plasmid. GFP-positive cells were isolated by FACS and immunoblotted with the indicated antibodies. **c**, The activity of an AP-1 reporter gene was measured in serum-starved or TPA-stimulated NIH 3T3 cells following cotransfection with RKIP antisense (pAS-C143) or empty vectors.

prevent MEK phosphorylation by MEKK-1 (Fig. 4c); and (3) did not interfere with Raf-1 autophosphorylation or phosphorylation of myelin basic protein (MBP) by Raf-1 (Fig. 4d). These data indicate that RKIP is a very selective inhibitor that specifically blocks activation of MEK by Raf.

In vitro, RKIP disrupted the physical interaction between Raf-1 and MEK, which is required for MEK phosphorylation¹², and behaved like a competitive inhibitor for MEK (data not shown). Therefore, we investigated whether this mechanism also operated in cells. The downregulation of endogenous RKIP expression by the pAS-C143 antisense vector substantially enhanced phosphorylation of MEK on activation-specific sites (Fig. 5a). Similarly, microinjection

of RKIP antibodies enhanced ERK activation in NIH 3T3 cells (Fig. 5b). In a complementary approach, RKIP was over-expressed. Cotransfection of RKIP had only a small influence on the activation of Raf-1 by TPA, but strongly inhibited the activation of MEK in a dose-dependent fashion (Fig. 5c). EGF produced the same results (data not shown). RKIP overexpression also downregulated the activation of ERKs by the *v-Ras* or *v-Src* oncogenes (Fig. 5d). Cotransfection of increasing amounts of an RKIP expression plasmid inhibited the BXB-induced activation of ERK in a dose-dependent manner. In contrast, RKIP did not affect activation by ERK by MEK-DD (Fig. 5e). These data confirm the *in vitro* results (Fig. 4), and show that RKIP regulates the ERK pathway primarily at the Raf/MEK interface *in vivo*.

To test whether the association between Raf and RKIP changes during mitogenic stimulation, we monitored the presence of RKIP in Raf-1 immunoprecipitates prepared from Rat-1 cells at different times after stimulation with serum (Fig. 6). The activation kinetics of the ERK pathway closely correlated with a decrease in RKIP coprecipitation. Furthermore, as the activity of the ERK pathway returned to basal levels at later times following mitogenic stimulation, the interaction between Raf-1 and RKIP returned to the level seen in quiescent cells.

What could be the physiological role of an inhibitor such as RKIP? Like the ERK pathway, RKIP is widely expressed. A quantification of Raf-1, MEK, ERK and RKIP protein levels in the cell lines used in this study showed a wide variation in RKIP expression relative to the kinases. The ratio Raf-1:MEK:ERK:RKIP was 1:1.6:2.4:14 in Rat-1; 1:1.4:3.5:27 in 208F; 1:0.7:9:4.2 in NIH 3T3; and 1:2.9:5.9:1.9 in COS-1 cells. Thus, at least in the three fibroblast cell lines, RKIP is abundant enough to be stoichiometrically relevant as an inhibitor. RKIP also colocalized with Raf-1 in Ras-transformed cells (Fig. 1d), indicating that an appreciable fraction of Raf-1 and its inhibitor RKIP may remain associated even under conditions that promote Raf-1 activation. This may in part explain the observation that only a small fraction of Raf-1 can be activated¹³. We propose that RKIP could function like a rheostat that sets the sensitivity threshold for the activation of the Raf/MEK/ERK pathway. A quantitative analysis of the activation kinetics of the ERK pathway showed that this cascade operates like a switch

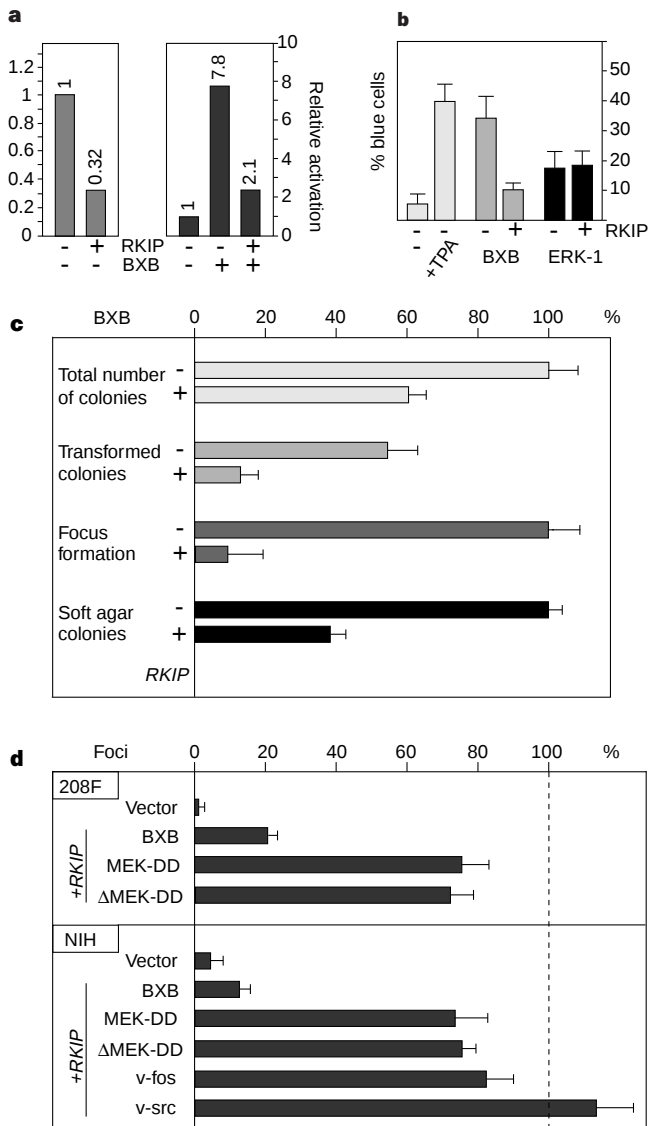


Figure 3 RKIP inhibits Raf-induced AP-1 activation and transformation. **a**, RKIP reduces basal and BXB-induced AP-1 activity in NIH 3T3 cells cotransfected with a 3xTRE-CAT reporter and the indicated expression plasmids. **b**, RKIP blocks BXB- but not ERK-induced AP-1 activation. Rat-1 cells were co-microinjected with a 4xTRE-lacZ reporter and the indicated expression vectors. **c**, RKIP inhibits Raf-dependent proliferation and transformation. NIH 3T3 cells were transfected with BXB, alone or together with RKIP (linked to neo). G418-resistant colonies were counted and scored for morphological transformation. Aliquots of the same transfection were allowed to grow to confluency without drug and were scored for focus formation. A BXB-transformed cell line was infected with LXSH-RKIP retrovirus or LXSH (hygromycin resistant) and seeded in soft agar in the presence of hygromycin. **d**, RKIP does not inhibit transformation by *v-fos*, *v-src* or mutationally activated MEK¹¹ in 208F or NIH cells. Data are expressed as reduction in focus formation relative to cotransfection with empty vector (set to 100%).

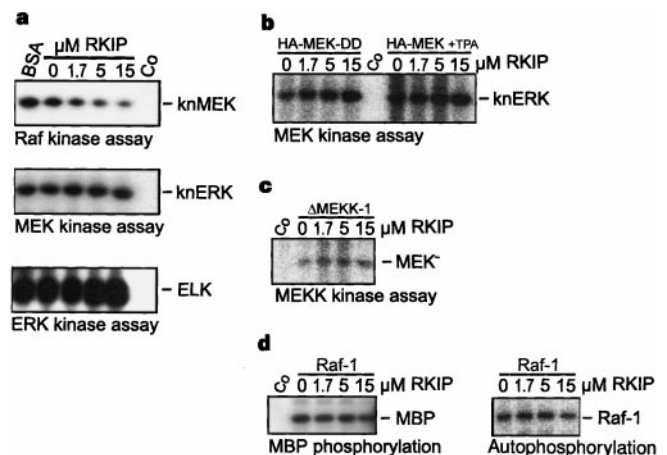


Figure 4 RKIP specifically blocks MEK phosphorylation by Raf-1. **a**, Effect of RKIP on the activation steps of the Raf/MEK/ERK cascade reconstituted *in vitro* with purified recombinant proteins. BSA, 15 μ M bovine serum albumin; Co, substrate alone; kn, kinase-negative mutant. **b**, RKIP does not inhibit activated MEK. HA-MEK-DD or HA-MEK-1 (ref. 11) expressed in COS-1 cells was immunoprecipitated with anti-HA antibodies from serum-starved cells or TPA-treated cells, respectively, and assayed for kinase activity. **c**, RKIP does not inhibit MEK phosphorylation by MEKK-1. Δ MEKK-1 (ref. 26) was immunoprecipitated from transiently transfected COS-1 cells and used to phosphorylate knMEK. **d**, RKIP does not inhibit Raf-1 autophosphorylation or phosphorylation of MBP.

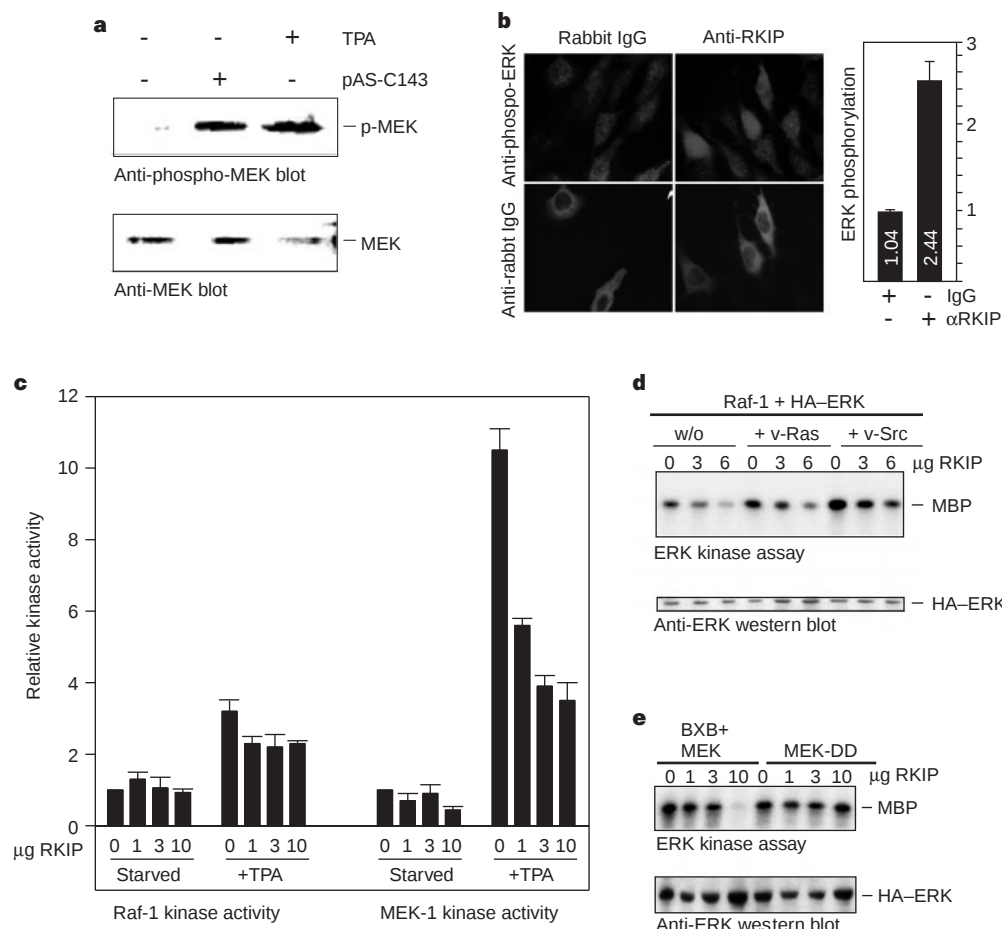


Figure 5 RKIP regulates MEK and ERK activation *in vivo*. **a**, RKIP downregulation activates MEK. NIH 3T3 cells were cotransfected with GFP and the RKIP antisense plasmid, pAS-C143. GFP-positive cells were FACS sorted and immunoblotted with the indicated antisera. **b**, RKIP antibody microinjection enhances ERK activation. Quiescent NIH 3T3 cells were microinjected with anti-RKIP or control IgG and stimulated with 10 ng ml⁻¹ TPA for 30 min. ERK activation was visualized with a monoclonal anti-phospho-ERK antibody (Sigma) and quantified densitometrically. **c**, RKIP inhibits MEK-1 activation. COS-1 cells were transiently transfected with HA-MEK and increasing amounts of RKIP expression

vectors. Serum-starved cells were stimulated with 100 ng ml⁻¹ TPA for 20 min, and the kinase activities of Raf-1 and HA-MEK immunoprecipitates were measured. **d**, RKIP inhibits stimulation of ERK by v-Ras and v-Src. COS-1 cells were transfected with the indicated expression plasmids plus increasing amounts of RKIP. HA-ERK-2 was immunoprecipitated and assayed with MBP. **e**, RKIP inhibits ERK activation by BXB, but not by MEK-DD. COS-1 cells were transfected with the indicated expression vectors and the kinase activity of HA-ERK immunoprecipitates was examined.

that suppresses background noise but strongly amplifies signals exceeding a certain threshold¹⁴. Overexpression of RKIP raises this threshold, whereas downregulation of RKIP lowers it. As the amplitude, kinetics and overall duration of ERK activity are known to vary between biological responses such as cell-cycle arrest, transformation, mitogenesis and differentiation^{1,15-18}, RKIP is expected to exert a profound influence on these parameters. □

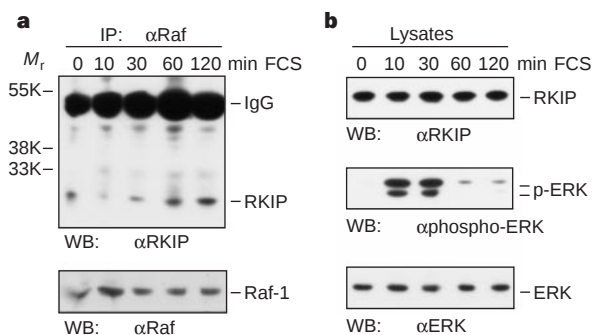


Figure 6 During mitogenic stimulation RKIP binding to Raf-1 decreases. Serum-starved Rat-1 cells were treated with 20% fetal calf serum for the indicated times. **a**, Raf-1 immunoprecipitates were immunoblotted for associated RKIP. **b**, Cell lysates were examined for RKIP and ERK expression. ERK activation was monitored with a phospho-ERK specific antibody.

Methods

Plasmids and protein expression

The rat RKIP cDNA¹⁹ was cloned (1) into pcDNA3 to make p353/RKIP; (2) into pCMV5 with a triple HA-tag at the amino-terminus; and (3) into pGEX-KG to make GST-RKIP. pAS-C143 encompasses RKIP nucleotides 1-429 cloned into pCMVori in antisense orientation. pCMVori contains the CMV promoter, polylinker and polyadenylation sequences from pCMV5 inserted into pUCori upstream of the polyoma virus core origin²⁰. 6xHis-tagged MEK- and GST-fusion proteins were expressed and purified as described²¹. RKIP of >95% purity was prepared from GST-RKIP by thrombin cleavage²² and subsequent FPLC separation over Superose 12.

In vitro binding assays

These contained 1-5 μ g of GST-fusion protein immobilized on glutathione sepharose beads and 0.5-5 μ g purified recombinant protein in PBS supplemented with 10% bovine serum as a nonspecific competitor. Sf-9 cell lysates were used as a source of Raf proteins²¹. After incubation for 1 h at 4 °C the samples were washed 4 times with PBS, resolved by SDS-PAGE and blotted. The blots were developed using ECL (Amersham).

Immunoprecipitation

For co-precipitation experiments of endogenous proteins, 2×10^7 Rat-1 cells were lysed by sonication in PBS, and the immunoprecipitates were washed 4 times with PBS. Otherwise cells were lysed as described²¹. The antibodies used were crafVI, a peptide antibody against the 12 carboxy-terminal amino acids of Raf-1 (ref. 21); a Raf monoclonal antibody to the regulatory domain (Transduction Laboratories); anti-MEK H8 (Santa Cruz); anti-RKIP raised in rabbits immunized with purified GST-RKIP; anti-HA, 12CA5 monoclonal antibody; monoclonal anti-phospho-ERK (Sigma) and polyclonal anti-phospho-MEK antibodies (New England Biolabs); and anti-GST (Pharmacia).

Kinase assays

These were done as described²¹. For *in vitro* reconstruction of the Raf/MEK/ERK cascade, activated Raf-1 was generated by co-expressing GST-Raf-1 with v-Ras and Lck in Sf-9 cells and collected on glutathione-sepharose beads²¹. Subsequent thrombin cleavage released Raf-1, which was fully active and >90% pure. To activate MEK and ERK *in vitro*, 20 ng of activated Raf-1 was incubated with 40 ng of purified His/MEK-1 and 250 ng of GST-ERK-2 in Raf kinase buffer containing 20 μ M ATP for 20 min at 30 °C. To measure kinase activities at individual steps, the respective downstream components were omitted. The activation reactions were diluted into 50 μ l Raf kinase buffer²¹ containing 20 μ M ATP to yield equimolar concentrations of the kinases to be assayed and incubated with increasing amounts of purified RKIP on ice for 10 min. Then, 2 μ Ci [³²P]- γ -ATP and recombinant substrates were added and incubated for 20 min at 30 °C. As substrates, we used 200 ng kinase negative His/MEK-1 for Raf, 1 μ g kinase negative GST-ERK for MEK and 1 μ g GST-ELK (New England Biolabs) for ERK. In some assays 1 μ g GST-MEK was used as the Raf-1 substrate with identical results.

Transfections

COS-1 cells were transfected as described²³ with 2 μ g of HA-ERK-2, BXB, MEK and MEK-DD plasmids and the indicated amounts of p353/RKIP. The total amount of transfected DNA was kept constant using the appropriate vectors as carrier DNA. NIH 3T3 and 208F cells were transfected in 6-well plates with 1 μ g of pCMV5-BXB and 3 μ g of p353/RKIP using Superfect (Qiagen). For RKIP downregulation experiments, NIH 3T3 cells were transiently cotransfected using lipofectamine with 0.5 μ g of pHACT²⁰ and 1.5 or 3 μ g RKIP antisense expression vector (pAS-C143) or control vector (pCMVori) as indicated. pHACT expresses a truncated polyoma large-T construct with origin-binding activity, but does not bind Rb or p53, and boosts the expression of pAS-C143 to high levels. In addition, 0.1 μ g of an AP1-Luc reporter was transfected for reporter-gene assays. 48 hours after transfection, cells were serum starved for 20 h and either left untreated or treated with TPA (200 ng ml⁻¹) or serum for 5 h before being collected. Cells were lysed and cell extracts were used for immunoblotting or assayed for luciferase activity. For the green fluorescent protein (GFP) sorting experiments, 5×10^6 NIH 3T3 cells were electroporated with either 100 μ g pCMVori, 50 μ g pCMV-GFP and 50 μ g pHACT, or 100 μ g pAS-C143, 50 μ g CMV-GFP and 50 μ g CMV-HAC. Two days later cells were trypsinized and sorted for green fluorescent cells by preparative FACS. 100,000 GFP-positive cells were lysed in SDS-gel sample buffer and immunoblotted.

Microinjection

Microinjection of antibodies and reporter genes was done as described^{24,25}. The RKIP antiserum was purified over a GST-RKIP affinity column. Cells were stained with an activation-specific anti-phospho-ERK monoclonal antibody (New England Biolabs). ERK phosphorylation was quantified by densitometry. For this purpose areas with micro-injected cells were randomly photographed, and the staining intensity of whole individual cells was measured using the PbAS software.

Received 8 April; accepted 8 July 1999.

1. Ferrell, J. E. Jr MAP kinases in mitogenesis and development. *Curr. Top. Dev. Biol.* **33**, 1–60 (1996).
2. Morrison, D. K. & Cutler, R. E. The complexity of Raf-1 regulation. *Curr. Opin. Cell Biol.* **9**, 174–179 (1997).
3. Moodie, S. A., Willumsen, B. M., Weber, M. J. & Wolfman, A. Complexes of Ras/GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260**, 1658–1661 (1993).
4. Schaeffer, H. J. et al. MP1: A MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science* **281**, 1668–1671 (1998).
5. Bruder, J. T., Heidecker, G. & Rapp, U. R. Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. *Genes Dev.* **6**, 545–556 (1992).
6. Li, S. et al. Regulation of Raf-1 kinase activity by the 14-3-3 family of proteins. *EMBO J.* **14**, 685–696 (1995).
7. Schoentgen, F. & Jolles, P. From structure to function: possible biological roles of a new widespread protein family binding hydrophobic ligands and displaying a nucleotide binding site. *FEBS Lett.* **369**, 22–26 (1995).
8. Kortjenann, M., Thomae, O. & Shaw, P. E. Inhibition of v-raf-dependent c-fos expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. *Mol. Cell. Biol.* **14**, 4815–4824 (1994).
9. Rapp, U. R., Troppmair, J., Beck, T. & Birrer, M. J. Transformation by Raf and other oncogenes renders cells differentially sensitive to growth inhibition by a dominant negative c-jun mutant. *Oncogene* **9**, 3493–3498 (1994).
10. Kolch, W. et al. Raf revertant cells resist transformation by non-nuclear oncogenes and are deficient in the induction of early response genes by TPA and serum. *Oncogene* **8**, 361–370 (1993).
11. Catlings, A. D., Schaeffer, H. J., Reuter, C. W., Reddy, G. R. & Weber, M. J. A proline-rich sequence unique to MEK1 and MEK2 is required for raf binding and regulates MEK function. *Mol. Cell. Biol.* **15**, 5214–5225 (1995).
12. Kolch, W. et al. Inhibition of Raf-1 signaling by a monoclonal antibody, which interferes with Raf-1 activation and with Mek substrate binding. *Oncogene* **13**, 1305–1314 (1996).

13. Hallberg, B., Rayter, S. I. & Downward, J. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. *J. Biol. Chem.* **269**, 3913–3916 (1994).
14. Ferrell, J. E. Jr Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends Biochem. Sci.* **21**, 460–466 (1996).
15. Tombes, R. M. et al. The mitogen-activated protein (MAP) kinase cascade can either stimulate or inhibit DNA synthesis in primary cultures of rat hepatocytes depending upon whether its activation is acute/phasic or chronic. *Biochem. J.* **330**, 1451–1460 (1998).
16. Marshall, C. J. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179–185 (1995).
17. Sewing, A., Wiseman, B., Lloyd, A. C. & Land, H. High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. *Mol. Cell. Biol.* **17**, 5588–5597 (1997).
18. Woods, D. et al. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. *Mol. Cell. Biol.* **17**, 5598–5611 (1997).
19. Grandy, D. K. et al. Purification, cloning, and tissue distribution of a 23-kDa rat protein isolated by morphine affinity chromatography. *Mol. Endocrinol.* **4**, 1370–1376 (1990).
20. Gjurup, O. V., Rose, P. E., Holman, P. S., Bockus, B. J. & Schaffhausen, B. S. Protein domains connect cell cycle stimulation directly to initiation of DNA replication. *Proc. Natl Acad. Sci. USA* **91**, 12125–12129 (1994).
21. Häfner, S. et al. Mechanism of inhibition of Raf-1 by protein kinase A. *Mol. Cell. Biol.* **14**, 6696–6703 (1994).
22. Guan, K. L. & Dixon, J. E. Eucaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**, 262–267 (1991).
23. Mischak, H. et al. Negative regulation of Raf-1 by phosphorylation of serine 621. *Mol. Cell. Biol.* **16**, 5409–5418 (1996).
24. Lavbinsky, R. M. et al. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc. Natl Acad. Sci. USA* **95**, 2920–2925 (1998).
25. Rose, D. W., McCabe, G., Feramisco, J. R. & Adler, M. Expression of c-fos and AP-1 activity in senescent human fibroblasts is not sufficient for DNA synthesis. *J. Cell Biol.* **119**, 1405–1411 (1992).
26. Minden, A. et al. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* **266**, 1719–1723 (1994).

Acknowledgements

We thank M. Karin, M. Weber, F. Hofer, P. Shaw, M. Baccarini and B. Schaffhausen for various expression plasmids; D. K. Grandy for the full-length rat cDNA homologue of RKIP; and L. Black for help with microinjections. We appreciate critical reading and helpful comments by members of the Beatson laboratories. This work was in part supported by grants of the AICR, DFG and the Sanders Stiftung to W.K. and H.M. and by an NIH grant to J.M.S.

Correspondence and requests for materials should be addressed to W.K. (e-mail: wkolch@beatson.gla.ac.uk) or J.M.S. (e-mail: john_sedivy@brown.edu).

Saccharomyces cerevisiae telomerase is an Sm small nuclear ribonucleoprotein particle

Anita G. Seto*, Arthur J. Zaugg*, Suzanne G. Sobel†, Sandra L. Wolin† & Thomas R. Cech*

* Department of Chemistry and Biochemistry and Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado 80309-0215, USA

† Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06510, USA

Activation of the chromosome end-replicating enzyme telomerase can greatly extend the lifespan of normal human cells¹ and is associated with most human cancers². In all eukaryotes examined, telomerase has an RNA subunit³, a conserved reverse transcriptase subunit⁴ and additional proteins^{5,6}, but little is known about the assembly of these components. Here we show that the *Saccharomyces cerevisiae* telomerase RNA⁷ has a 5'-2,2,7-trimethylguanosine (TMG) cap and a binding site for the Sm proteins, both hallmarks of small nuclear ribonucleoprotein particles (snRNPs) that are involved in nuclear messenger RNA splicing^{8,9}. Immunoprecipitation of telomerase from yeast extracts shows that Sm proteins are assembled on the RNA and that most or all of the telomerase activity is associated with the Sm-containing complex. These data support a model in which telomerase RNA is transcribed by RNA polymerase II (ref. 10) and 7-methylguanosine-capped,

binds the seven Sm proteins, becomes TMG-capped and picks up the other protein subunits. We conclude that the functions of snRNPs assembled by this pathway are not restricted to RNA processing, but also include chromosome telomere replication.

The nature of the 5' end of an RNA often provides a clue to its biogenesis pathway. The presence of a 5'-TMG cap on telomerase RNA (*TLC1*) was tested by immunoprecipitation of yeast total cellular RNA with anti-TMG antibodies. Northern blot hybridization (Fig. 1) showed that *TLC1* RNA was immunoprecipitated, as was U1 snRNA, which is known to have a TMG cap¹¹ (32% and 55%, respectively, of the input RNA were bound to the antibody). In contrast, uncapped 5.8S ribosomal RNA was recovered predominantly in the supernatant (only 3.4% bound), providing evidence for the specificity of the immunoprecipitation. Immunoprecipitated RNA from a strain carrying a deletion of the gene for telomerase RNA (*tlc1Δ*) contained U1 and 5.8S RNAs but not *TLC1* RNA, as expected. Yeast telomerase RNA consists of a major poly(A)⁻ species and a short-lived poly(A)⁺ transcript that is thought to be its precursor¹⁰; the poly(A)⁻ fraction of the telomerase RNA was preferentially enriched on the anti-TMG beads relative to the poly(A)⁺ fraction (data not shown).

The presence of a TMG cap provided the impetus for searching for additional snRNP features of telomerase RNA. Inspection of the primary sequence of *TLC1* revealed a sequence matching that of the known consensus for Sm protein binding (AU₅₋₆Gpu) and identical to the Sm site in yeast U4 snRNA¹². This sequence is near the 3' end of *TLC1* RNA, similar to its location in other yeast Sm snRNAs (Fig. 2a). To test the functional significance of this putative Sm site, low-copy-number plasmids carrying various mutations in *TLC1* were constructed and individually transformed into a *tlc1Δrad52⁻*-strain (Fig. 2a). Disruption of *RAD52* disables the recombination pathway, an alternative mechanism for telomere maintenance in yeast⁶, thereby enabling clear assessment of defects in telomerase activity.

Yeast strains with the *tlc1-Sm⁻* mutation, which replaces the putative Sm site with an unrelated sequence, or two less severe mutations, *tlc1-Sm2T* and *tlc1-Sm4C5C*, had markedly decreased levels of telomerase RNA (Fig. A in Supplementary Information). The deadenylated mature form was not visible by northern blot analysis. The residual telomerase RNA was the minor polyadenylated species, as shown by its enrichment on an oligo(dT) cellulose column (Fig. 2b). The *tlc1-Sm⁻* strain showed a biphasic growth pattern on plates: formation of normal-sized colonies was greatly reduced after about 100–125 generations (compared with 75–100 generations for senescence of the *tlc1Δ* strain), but the cells later recovered and grew normally (Fig. 2a). *Tlc1-Sm2T* and *tlc1-Sm4C5C* showed no growth phenotype. Presumably the residual levels of the mutant telomerase RNAs were sufficient to support cell growth.

Telomere length is often more sensitive to alterations in telomerase function than is cell growth¹³. Southern blot analysis (Fig. 2c) of DNA from the *tlc1-Sm⁻* strain showed that the telomeres initially began to shorten and then were maintained at a length shorter than wild-type. This pattern of telomere shortening correlated with the observed growth phenotype and differed from that of the *tlc1Δ* strain, in which the telomeres continued to shorten over time until the cells stopped growing (after the fourth successive culture, see Fig. 2d). In *tlc1-Sm2T* and *tlc1-Sm4C5C*, the telomeres were shorter than wild-type and appeared to remain about the same length, perhaps with very gradual shortening over time. Mutant telomerase RNA levels remained barely detectable over the time-course of this experiment (see Supplementary Information, Fig. A). These results indicate that the Sm proteins may be involved in the stability and function of telomerase RNA.

To test for interaction between Sm proteins and telomerase, we used co-immunoprecipitation experiments. We constructed a strain in which the endogenous *SMD1* gene, encoding one of the core Sm proteins, was replaced with an allele containing an influenza haemagglutinin (HA) epitope tag at its carboxy terminus. This

variant of *SMD1* had been previously characterized as a fully functional allele with no overt phenotype when expressed from a low-copy-number plasmid¹⁴. This strain also carries a gene for the catalytic subunit of yeast telomerase, *Est2p*, with an amino-terminal Protein A tag integrated at its chromosomal locus. The tag has no significant effect on growth or telomerase function (K. L. Friedman and T.R.C., unpublished data). Extract prepared from this strain was immunoprecipitated for HA-Smd1p with an anti-HA antibody bound to Protein G-agarose beads. Northern blot analysis of RNA extracted from the beads (Fig. 3a) revealed that telomerase RNA co-immunoprecipitated with HA-Smd1p more efficiently than U1 snRNA, previously shown to associate with Sm proteins^{15,16}. (Percentages of input RNA bound were 12% and 5%, respectively.) U3 small nucleolar RNA, not known to bind Sm proteins¹¹, did not co-immunoprecipitate with HA-Smd1p (only 0.09% bound). When extract was prepared from either a wild-type strain or a strain expressing only ProA-Est2p, no appreciable telomerase RNA or U1 snRNA bound to the anti-HA beads, demonstrating the specificity of the co-immunoprecipitation for HA-tagged Smd1p (Fig. 3a).

A second yeast Sm protein, Smd3p, when HA-tagged and expressed from a low-copy-number plasmid¹⁴, also co-immunoprecipitated *TLC1* RNA (data not shown). Additionally, when expression of Smd1 protein was turned off in a strain in which transcription of *SMD1* is controlled by the galactose promoter¹⁴, telomerase RNA levels decreased over time with kinetics similar to those of U1 snRNA, whereas U6, a non-Sm-containing snRNA, was unaffected (see Supplementary Information, Fig. B). Collectively, these data indicate that the Smd1 and Smd3 proteins are physically associated with the telomerase RNA and are required for its accumulation.

To determine whether the *TLC1* RNA associated with Smd1p was part of a functional enzyme, the complex bound to anti-HA beads was assayed *in vitro* for telomerase activity. Upon incubation with a single-stranded telomeric oligonucleotide, dTTP, and [³²P]dGTP, the pattern of extension products characteristic of yeast telomerase^{4,17,18} was detected (Fig. 3b, lane 1). This activity was sensitive to treatment with RNase A, a diagnostic property of telomerase (data not shown). The supernatant from the first incubation still contained some residual telomerase activity that was reduced by successive passages over anti-HA beads (Fig. 3b, lanes 2 and 3). Immunoprecipitation of wild-type extract, lacking tagged proteins, gave no detectable telomerase activity (data not shown). In addition, HA immunoprecipitates of extracts made from a strain of yeast carrying a low-copy plasmid encoding HA-tagged Smd3p¹⁴ also contained active telomerase (data not shown). Therefore, yeast telomerase associated with two different Sm proteins has enzymatic activity.

It remained possible that there were two populations of active telomerase, one with and one without the Sm proteins. To test this

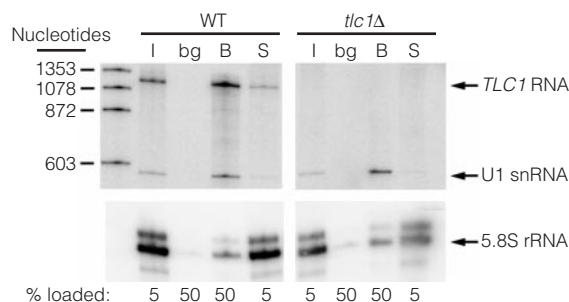


Figure 1 Identification of 5'-TMG cap on telomerase RNA. Northern blot of RNA fractions immunoprecipitated with agarose bead-immobilized anti-TMG antibodies: Input RNA (I), RNA bound to beads lacking antibody (bg), RNA bound to anti-TMG beads (B) and supernatant RNA (S). RNA was prepared from a wild-type (WT) strain and a strain deleted for *TLC1* (*tlc1Δ*), and probed with a random-primed [³²P]dCTP-labelled DNA copy of the *TLC1* gene and ³²P 5'-end-labelled oligonucleotides complementary to U1 and 5.8S. % loaded is the percentage of the total RNA from each experiment loaded in that lane. Markers in the left-most lane are end-labelled denatured φX174 DNA *Hae*III fragments.

possibility, Smd1p-containing complexes were significantly depleted from extracts of the strain containing both HA-tagged Smd1p and ProA-tagged Est2p by three rounds of incubation with anti-HA beads. The supernatant largely depleted of Smd1p was then incubated with IgG beads (which bind ProA–Est2p) to pull down any remaining telomerase complex, and the beads were assayed for telomerase activity (Fig. 3c, lane 2). In a parallel immunoprecipitation, an identical extract was treated with three rounds of control agarose beads; telomerase was then bound to IgG beads that were assayed for activity (Fig. 3c, lane 1). Quantification showed that the fraction of active telomerase containing the Sm protein is at least $77 \pm 7\%$ (average $\pm$ range of four experiments; given the incomplete nature of immunodepletion, this is a minimum estimate). These values were corrected for the small amount of telomerase activity lost nonspecifically on the anti-HA beads, as determined by preparing extract from a strain expressing only ProA–Est2p and subjecting it to three rounds of depletion by anti-HA or control beads followed by binding to IgG beads (Fig. 3c, lanes 3 and 4). An additional control showed that telomerase activity from wild-type extracts was not

bound to IgG beads (Fig. 3c, lanes 5 and 6). Western blot analysis revealed a reduction in Est2p after immunoprecipitation of the Smd1p-containing complex from the doubly-tagged strain (see Supplementary Information, Fig. C). These results indicate that at least the majority of telomerase in yeast is associated with Smd1p.

We have shown that the yeast telomerase RNA shares two key characteristics of the splicing snRNAs: capping by 5'-TMG and association with Sm proteins. Additionally, telomerase RNA has a polyadenylated species that is processed to a mature form¹⁰; minor polyadenylated fractions of U1 and U2 snRNAs have also been reported^{19,20}. To our knowledge, yeast telomerase is the only Sm-containing snRNP whose known function is not RNA processing,

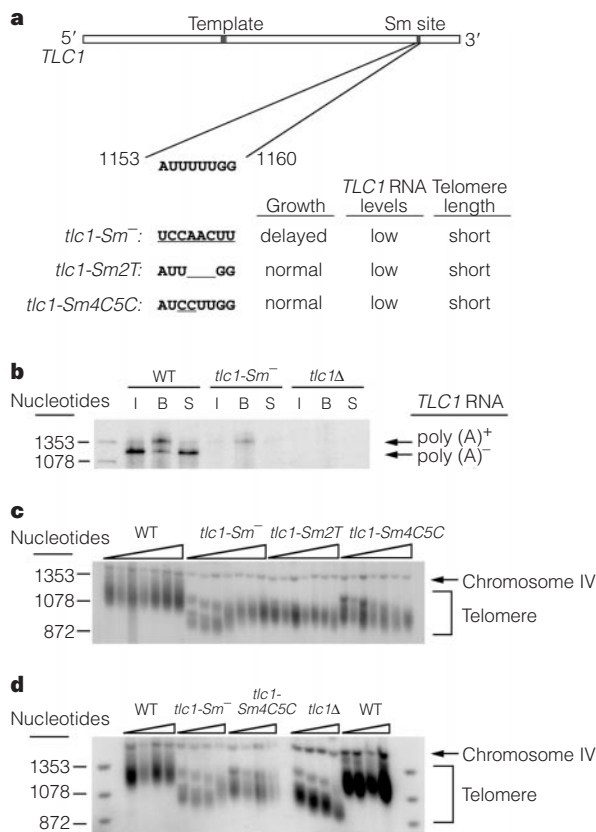


Figure 2 Characterization of mutants in the putative Sm protein binding site. **a**, The putative Sm site in *TLC1* RNA, mutations and deletions introduced in the site (underlined bases) and summary of observed phenotypes. **b**, *TLC1* RNA levels in Sm mutants were lower than wild-type and the remaining RNA was enriched on oligo(dT) cellulose. Northern blot of input (I), bound (B) and supernatant (S) fractions from oligo(dT) chromatography probed with *TLC1* gene probe. **c**, Telomere lengths of Sm site mutants were shorter than wild-type. Southern blot of genomic DNA digested with *XhoI*, hybridized with random-primed [³²P]dCTP-labelled DNA copies of telomeric sequence and a region in chromosome IV. *XhoI* cuts internal to the telomere in chromosomes containing Y' elements, producing a diffuse band of ~1–1.3 kilobases. Triangular wedges represent ~10–90 generations from seven successive cultures. The chromosome IV signal served as an internal loading control as well as a marker for measuring relative telomere lengths. **d**, Telomere shortening in Sm site mutants was not as severe as in the *tlc1Δ* strain. DNA was extracted from four successive cultures, ~10–50 generations. The *tlc1Δ* strain did not grow past the fourth culture. Wild-type DNA on the left was grown and prepared in parallel with *tlc1-Sm⁻* and *tlc1-Sm4C5C* DNA; wild-type on the right was from the same experiment as *tlc1Δ*.

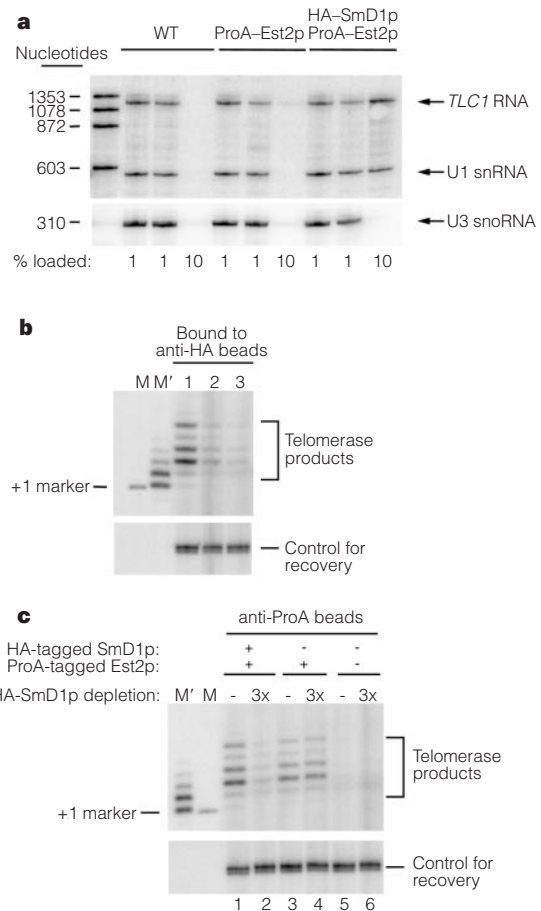


Figure 3 HA-tagged Smd1 protein co-immunoprecipitates *TLC1* RNA and *in vitro* telomerase activity. **a**, Co-immunoprecipitation of *TLC1* RNA with HA–Smd1p. Extract was prepared from a wild-type (WT) strain, a singly tagged strain expressing only ProA–Est2p, or a doubly tagged strain expressing both ProA–Est2p and HA–Smd1p. RNA that co-immunoprecipitated on anti-HA antibody beads from these strains was analysed by northern blot and probed with the *TLC1* gene probe and U1 and U3 oligonucleotide probes. I, input RNA; S, RNA in supernatant; B, RNA bound to beads. **b**, Co-immunoprecipitation of telomerase activity with HA–Smd1p. Extract prepared from a strain expressing HA-tagged Smd1p and ProA–Est2p was incubated with anti-HA antibody beads and the complexes on the beads were assayed for *in vitro* telomerase activity. Lane 1, telomerase activity precipitated on anti-HA beads; lanes 2 and 3, activity precipitated on a second and third passage of anti-HA beads, respectively. Control for recovery was a ³²P 5'-end-labelled 100-mer oligo added immediately after the reactions were stopped and visualized on the same gel. As markers, the 14-mer single-stranded telomeric primer was extended by one nucleotide with the addition of [³³P]ddTTP (M) or converted to a ladder with [³²P]dTTP and terminal transferase (M'). **c**, Most of the active telomerase contains Smd1p. Extracts prepared from strains described in **a** were incubated with three sequential batches of anti-HA beads (3x) or control beads lacking anti-HA antibody (–). The supernatants from these immunoprecipitations were then incubated with IgG beads to bind ProA–Est2p, thereby collecting the remaining telomerase activity. IgG beads were assayed for *in vitro* telomerase activity.

but rather DNA replication. The telomerase complex containing the Sm proteins has *in vitro* telomerase activity and represents the majority of telomerase activity in extracts. We infer that the Sm proteins constitute seven new telomerase subunits; we have provided direct evidence only for D1 and D3, but their participation in a ring of no less than seven subunits²¹ makes it likely that the other five are also present in telomerase.

The human telomerase RNA also has a TMG cap but the RNA is not detected by northern analysis in anti-Sm immunoprecipitates (A.J.Z., S.L.W. and T.R.C., data not shown; S. Lichtsteiner and S. L. Weinrich, personal communication), although it remains possible that the Sm epitope is masked in a large complex. The human RNA has a 3' domain characteristic of small nucleolar RNPs²². Therefore, it remains to be seen whether the biogenesis of the human telomerase is different or shares features with the yeast enzyme.

We propose a model for the biogenesis of yeast telomerase. The RNA subunit is transcribed by RNA polymerase II accompanied by 7-methyl G capping and polyadenylation¹⁰. The poly(A) tail is then removed by 3'-processing, which appears to require Sm protein binding, as shown by the fact that the poly(A)⁺ subfraction accumulates in the Sm-site RNA mutants. Binding of the Sm proteins to the Sm site also promotes the hypermethylation of the 5' cap to a trimethyl-G. In vertebrates, the U1, U2, U4 and U5 snRNAs are exported to the cytoplasm, where they bind Sm proteins, gain a TMG cap and are then re-imported into the nucleus²³. If such a cytoplasmic phase of the pathway occurs in yeast, it might provide an advantageous location for assembly of the telomerase complex, as the catalytic subunit (Est2p) and other telomerase proteins (such as Est1p²⁴ and Est3p^{6,13}) are synthesized in the cytoplasm. However, because shuttling of snRNAs between the nucleus and cytoplasm has not been shown in budding yeast, the yeast telomerase could be assembled entirely in the nucleus, where it ultimately functions. □

Methods

S. cerevisiae strains and manipulations

Strain TCy43 ($\Delta tlc1::LEU2$, $rad52::LEU2$, pl-TLC1⁺-LYS2⁺-CEN) was transformed with either pSD107 [CEN, TRP1, TLC1], pAS500 [CEN, TRP1, $tlc1\Delta$], pAS501 [CEN, TRP1, $tlc1-sm^+$], pAS502 [CEN, TRP1, $tlc1-sm2T$] or pAS503 [CEN, TRP1, $tlc1-sm4C5C$], and then grown on α -aminoacidate to counterselect plasmid pl-TLC1⁺-LYS2⁺-CEN as described^{25,26}. The construct for the integration of the haemagglutinin epitope tag into the SMD1 locus was made by ligating PCR fragments corresponding to nucleotides 55–452 of the SMD1 open reading frame of plasmid pGAL::SMD1HA¹⁴ and nucleotides 447–907 of the downstream flanking region of SMD1 (amplified from TVL268, see below) into plasmid pRS306. The correct sequence of the entire region was confirmed with automated sequencing. Integration of HA-SMD1 was performed by the pop-in/pop-out method²⁷ into the wild-type strain TVL268 (V. Lundblad) and into YKF103 (details of strain construction to be described elsewhere; K. L. Friedman and T.R.C., unpublished data). Correct integration was confirmed by PCR. For additional strain information and plasmid construction, see Methods in Supplementary Information.

TMG immunoprecipitations and poly(A)⁺ isolation of RNA

See Methods in Supplementary Information.

Measurements of telomere length

See Methods in Supplementary Information.

Co-immunoprecipitation experiments

Yeast cultures were grown to 0.9–1.5 OD (600 nm). Extracts were prepared by a glass-bead lysis method in PMG-150 (10 mM phosphate pH 6.8, 1 mM MgCl₂, 10% glycerol, 150 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 1 U μ l⁻¹ RNasin (Promega)). The low salt, low pH buffer was used to decrease the affinity of the Protein A tag to the anti-HA antibody (mouse IgG)²⁸. For HA immunoprecipitation, anti-HA antibody beads were prepared by overnight incubation of 250–350 μ g of monoclonal antibody HA.11 (Babco) to 200 μ l of packed protein G Plus-Agarose (Oncogene). Beads were washed extensively with PBS. To 1.5–2.5 mg of whole cell extract, 20 μ l of packed anti-HA-beads was added. After binding for 4–12 h, the beads were washed extensively with PMG-150. ProA-Est2p immunoprecipitations were performed by incubation of 1.5–2.5 mg extract with IgG Sepharose beads (Amersham Pharmacia) for 12 h. RNA associated with the antibody-antigen complexes on the anti-HA or IgG beads was isolated as described¹⁴. RNA was isolated from extract by treatment with 0.4 mg ml⁻¹ proteinase K for 15 min at 37 °C followed by phenol/chloroform extraction and precipitation with 0.3 M NaOAc and 3

volumes cold 100% EtOH. RNA was then analysed by northern blot as described¹⁰.

Telomerase activity assay.

Telomerase complex bound to 5 μ l packed HA or IgG beads was incubated in telomerase assay buffer (40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 0.5 mM spermidine, 0.5 mM DTT, 2.5 μ M substrate oligo (5'-GTGTGGTGTGTGGG-3'), 100 μ M dTTP, 1.70 μ M [α -³²P]dGTP) for 20 min at 30 °C. Alternatively, reactions were performed with 100 μ M ddGTP and 1.70 μ M [α -³²P]dTTP (data not shown). To the reaction, ³²P-5'-end-labelled 100-mer was added as a loading control along with 1 μ l glycogen. Products were phenol/chloroform extracted, then ethanol precipitated with 7.5 M NH₄OAc and 2.5 volumes cold 100% ethanol. Products were resolved on a 12% PAGE-urea sequencing gel and visualized with a Molecular Dynamics PhosphorImager.

Received 26 April; accepted 15 July 1999.

1. Bodnar, A. G. *et al.* Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352 (1998).
2. Kim, N. W. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2103 (1994).
3. Greider, C. W. & Blackburn, E. H. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* **337**, 331–337 (1989).
4. Lingner, J. *et al.* Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* **276**, 561–567 (1997).
5. Bryan, T. M. & Cech, T. R. Telomerase and the maintenance of chromosome ends. *Curr. Opin. Cell Biol.* **11**, 318–324 (1999).
6. Nugent, C. I. & Lundblad, V. The telomerase reverse transcriptase: components and regulation. *Genes Dev.* **12**, 1073–1085 (1998).
7. Singer, M. S. & Gottschling, D. E. TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* **266**, 404–409 (1994).
8. Lührmann, R., Kastner, B. & Bach, M. Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochim. Biophys. Acta* **1087**, 265–292 (1990).
9. Mattaj, I. W. Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. *Cell* **46**, 905–911 (1986).
10. Chapon, C., Cech, T. R. & Zaug, A. J. Polyadenylation of telomerase RNA in budding yeast. *RNA* **3**, 1337–1351 (1997).
11. Yu, Y.-T., Scharl, E. C., Smith, C. M. & Steitz, J. A. In *The RNA World* (eds Gesteland, R. F., Cech, T. R. & Atkins, J. F.) 487–524 (Cold Spring Harbor Lab. Press, Cold Spring Harbor, New York, 1999).
12. Jones, M. H. & Guthrie, C. Unexpected flexibility in an evolutionarily conserved protein-RNA interaction: genetic analysis of the Sm binding site. *EMBO J.* **9**, 2555–2561 (1990).
13. Lendvay, T. S., Morris, D. K., Sah, J., Balasubramanian, B. & Lundblad, V. Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional EST genes. *Genetics* **144**, 1399–1412 (1996).
14. Roy, J., Zheng, B., Rymond, B. C. & Woolford, J. L. Jr Structurally related but functionally distinct yeast Sm D core small nuclear ribonucleoprotein particle proteins. *Mol. Cell. Biol.* **15**, 445–455 (1995).
15. Pettersson, L., Hinterberger, M., Mimori, T., Gottlieb, E. & Steitz, J. A. The structure of mammalian small nuclear ribonucleoproteins: identification of multiple protein components reactive with anti-(U1)RNP and anti-Sm autoantibodies. *J. Biol. Chem.* **259**, 5907–5914 (1984).
16. Neubauer, G. *et al.* Identification of the proteins of the yeast U1 small nuclear ribonucleoprotein complex by mass spectrometry. *Proc. Natl Acad. Sci. USA* **94**, 385–390 (1997).
17. Cohn, M. & Blackburn, E. H. Telomerase in yeast. *Science* **269**, 396–400 (1995).
18. Prescott, J. & Blackburn, E. H. Telomerase RNA mutations in *Saccharomyces cerevisiae* alter telomerase action and reveal nonprocessivity *in vivo* and *in vitro*. *Genes Dev.* **11**, 528–540 (1997).
19. Seipelt, R. L., Zhang, B., Asuru, A. & Rymond, B. U1 snRNA is cleaved by RNase III and processed through an Sm site-dependent pathway. *Nucleic Acids Res.* **27**, 587–595 (1999).
20. Elela, S. A. & Ares, M. Jr Depletion of yeast RNase III blocks correct U2 3' end formation and results in polyadenylated but functional U2 snRNA. *EMBO J.* **17**, 3738–3746 (1998).
21. Kambach, C. *et al.* Crystal structures of two Sm protein complexes and their implications for the assembly of the spliceosomal snRNPs. *Cell* **96**, 375–387 (1999).
22. Mitchell, J. R., Cheng, J. & Collins, K. A box HACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. *Mol. Cell. Biol.* **19**, 567–576 (1999).
23. Mattaj, I. W. In *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles* (ed. Birnstiel, M. L.) 100–114 (Springer, Berlin, 1988).
24. Virta-Pearlman, G., Morris, D. K. & Lundblad, V. Est1 has the properties of a single-stranded telomere end-binding protein. *Genes Dev.* **10**, 3094–3104 (1996).
25. Rose, M. D., Winston, F. & Hieter, P. *Methods in Yeast Genetics. A Laboratory Manual* 177–186 (Cold Spring Harbor Lab. Press, Cold Spring Harbor, New York, 1990).
26. Schiestl, R. H. & Gietz, R. D. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**, 339–346 (1989).
27. Scherer, S. & Davis, R. W. Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. *Proc. Natl Acad. Sci. USA* **76**, 4951–4955 (1979).
28. Ey, P. L., Prowse, S. J. & Jenkin, C. R. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using Protein A-sepharose. *Immunochemistry* **15**, 429–436 (1978).

Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) as paper copy from the London editorial offices of Nature.

Acknowledgements

We thank K. Friedman, D. Gottschling, and B. Rymond for yeast plasmids and strains; R. Weilbaecher and V. Lundblad for their telomerase activity assay protocol; and the Cech lab and J. Goodrich for discussions. This work was supported by grants from the NIH to T.R.C. and S.L.W. T.R.C. and S.L.W. are investigators of the Howard Hughes Medical Institute.

Correspondence and requests for material should be addressed to T.R.C. (e-mail: thomas.cech@colorado.edu).

Coherent reaction dynamics in a bacterial cytochrome *c* oxidase

Ursula Liebl, Gérard Lipowski, Michel Négrerie, Jean-Christophe Lambry, Jean-Louis Martin & Marten H. Vos

INSERM U451, Laboratoire d'Optique Appliquée, Ecole Polytechnique-ENSTA, 91761 Palaiseau Cedex, France

Biological reactions in protein complexes involve structural dynamics spanning many orders of magnitude in time. In standard descriptions of catalysis by enzymes, the transition state between reactant and product is reached by thermal, stochastic motion. In the ultrashort time domain, however, the protein moiety and cofactor motions leading to altered conformations can be coherent rather than stochastic in nature^{1–4}. Such coherent motions may play a key role in controlling the accessibility of the transition state and explain the high efficiency of the reaction. Here we present evidence for coherent population transfer to the product state during an ultrafast reaction catalysed by a key enzyme in aerobic organisms. Using the enzyme cytochrome *c* oxidase *aa*₃ from the bacterium *Paracoccus denitrificans*, we have studied haem dynamics during the photo-initiated ultrafast transfer of carbon monoxide from haem *a*₃ to CuB by femtosecond spectroscopy. The ground state of the unliganded *a*₃ species is populated in a stepwise manner in time, indicating that the reaction is mainly governed by coherent vibrations (47 cm^{–1}). The reaction coordinate involves conformational relaxation of the haem group and we suggest that ligand transfer also contributes.

Cytochrome *c* oxidase *aa*₃ is the respiratory-chain enzyme that reduces molecular oxygen to water⁵. It contains two *a*-type haem groups: haem *a* is six-coordinated and mediates electron transfer from exogenous cytochrome *c* towards the active site; haem *a*₃, located near the copper atom CuB (Fe *a*₃–CuB ~5 Å), is five-coordinated and acts as the binding site for molecular oxygen and its reaction intermediates during its four-electron reduction. Haem *a*₃ also binds competitor ligands, including other diatomic gases like nitric oxide and carbon monoxide (Fig. 1a). NO and CO are physiologically generated and act as biological messengers. Because haem binds CO (and NO) tightly, the accessibility of these ligands to the haem *a*₃ in cytochrome *c* oxidase must be well controlled. In the bimetallic active site, CuB is probably crucial for regulating ligand traffic. Before binding to haem *a*₃, CO and O₂ bind to CuB intermediately^{6,7}; the reverse reaction, involving ligand transfer from haem *a*₃ out of the protein via CuB, can be studied using flash photolysis. Optical excitation of the *aa*₃–CO complex leads to breaking of the *a*₃–CO bond and then to the successive transfer and binding of CO to CuB. As this transfer occurs in less than 1 ps (ref. 8), which is much faster than would be expected for CO diffusion towards CuB, it may occur in a concerted manner with the motion of the haem pocket. Concomitant with CO movement in the active site, haem *a*₃ evolves from a low-spin state towards a five-coordinate high-spin state (in less than 5 ps; ref. 9), a process that presumably involves doming of the haem. The evolution of the electronic states of the haem groups on the timescale of CO transfer and haem rearrangement can be monitored by femtosecond spectroscopy¹⁰.

Figure 1b, c shows the spectral evolution in the haem Soret region upon impulsive excitation of fully reduced unliganded and CO-bound cytochrome *c* oxidase *aa*₃ from *P. denitrificans*. The excitation wavelength region was chosen to lie in the α -band to avoid complications due to low-frequency impulsive stimulated Raman signals, which greatly contribute to the coherent response upon excitation of haem proteins in the Soret band³. Oscillatory features

were not evident in the ground-state absorption region of the enzyme (result not shown). Excitation in the lowest-energy transition also minimizes the excess energy after photodissociation. Excitation at 590 nm excites both haem groups to a similar extent¹¹. The initial bleachings correspond to the ground-state absorption bands of the unliganded haem groups *a* and *a*₃ (~443 nm) and CO-liganded haem *a*₃ (~430 nm). We have focused on the kinetics associated with the formation of the unliganded ground state of haem *a*₃. On a timescale of longer than a few hundred femtoseconds, the overall spectral dynamics predominantly reflect the decay of the haem *a* excited state¹⁰ and are similar for the unliganded and CO-bound species.

Figure 2 shows the kinetics at 442 nm for reactions initiated from both the unliganded and CO-liganded enzymes. Comparison with exponential fit functions shows that the trace is modulated by an oscillatory feature for CO-liganded enzyme but not for unliganded enzyme, indicating that the oscillatory feature, which has a frequency of ~47 cm^{–1} (period, 700 fs), is related to haem *a*₃ dynamics upon photodissociation. A key observation is that this oscillatory feature appears in the absorption band of unliganded haem *a*₃, reflecting a modulation of the absorption of the unliganded product state. Two mechanisms that could give rise to such a modulation and generate characteristic spectroscopic features are coherent vibrational motion on the product-state potential-energy surface: in this case, the amplitude dependence of the oscillation should be bi-lobed, with a distinct disappearance at the centre of the band,

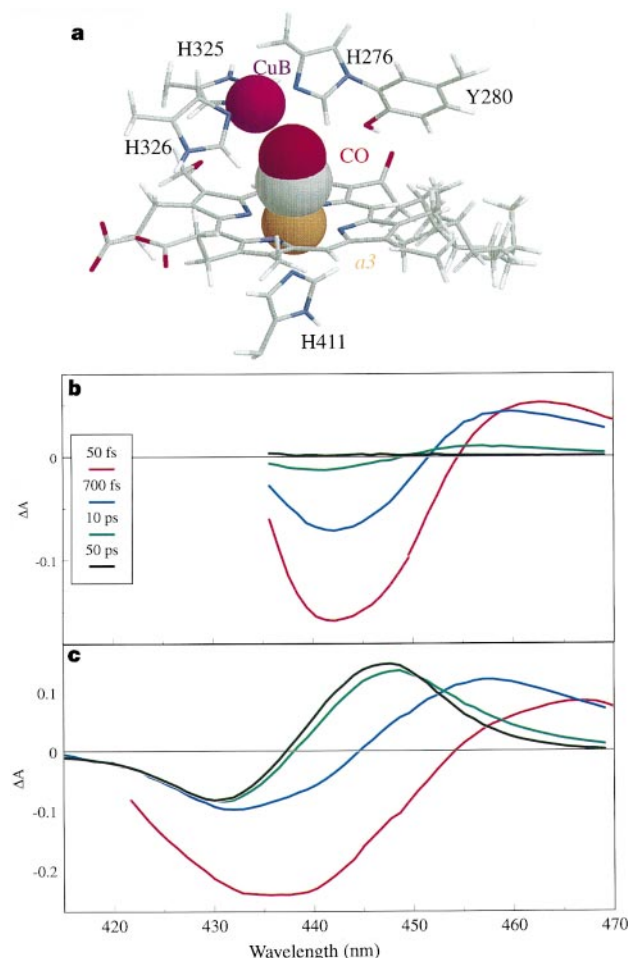


Figure 1 Overview of the binuclear site and transient spectroscopy of the haems. **a**, Model of the haem *a*₃–CuB site with CO bound to the haem *a*₃ iron atom. The yellow, magenta, grey and red spheres represent the van der Waals radius for *a*₃ iron atom, CuB and CO, respectively. **b**, **c**, Transient absorption spectra at various delay times of fully reduced non-liganded (**b**) and CO-bound (**c**) cytochrome *c* oxidase.

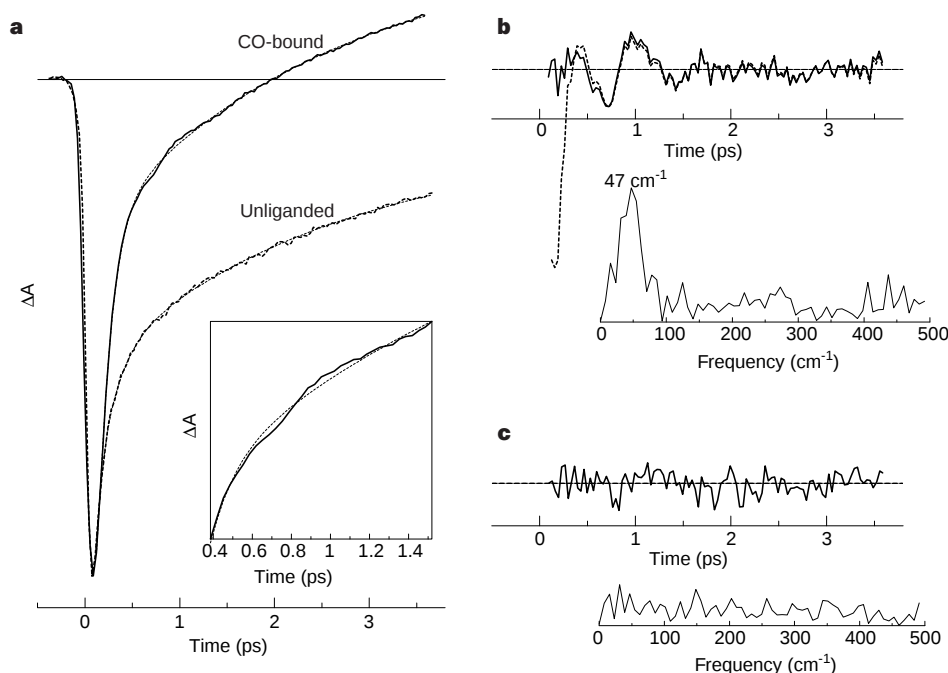


Figure 2 Kinetic analysis. **a**, Transient absorption kinetics at 442 nm of CO-bound (solid line, expanded in inset) and non-liganded (dashed line) cytochrome *c* oxidase and their exponential fits (thin dashed lines). **b**, **c**, Fit residuals (top traces, $\times 10$ expanded) and their Fourier transforms for CO-bound (**b**) and non-liganded (**c**) cytochrome *c* oxidase. The fit

procedure, starting at 50 fs (solid lines), naturally results in a minimization of the first oscillation. To illustrate this and the sensitivity of the residuals to the fit function, the residuals obtained with a fit starting at $t = 300$ fs extrapolated to earlier times (dashed line in **b**) are also shown.

accompanied by a sharp π -phase jump^{1,2,12}; and coherent (stepwise) population of the product state: in this case, the absolute amplitude dependence should trace the shape of the absorption band of the product state and the phase of the oscillations should be constant.

The amplitude dependence of the oscillations (Fig. 3b) is clear: a single maximum is observed at 440–445 nm, and the shape of the absolute amplitude dependence closely matches that of the expected absorption band of unliganded a_3 (refs 11, 13). Moreover, with the exception of the far-red side of the spectrum (see below), the phase of the oscillation has a constant value of π (Fig. 3a). These findings are inconsistent with the first mechanism, but represent the expected signature for the second scenario: after photodissociation, the population build-up of the product state, the unliganded ground state of haem a_3 , is modulated. In the simplest view, this implies that surface crossing towards the product state a_3 is coupled to coherent motion with a frequency of ~ 47 cm^{-1} and that it occurs in a stepwise manner (Fig. 4).

The direct precursor state of the unliganded ground state is the excited state a_3^* (Fig. 4b), the analogue of Hb_1^* in haemoglobin¹⁴. The breaking of the Fe–CO bond presumably occurs on the time-scale of the Fe–CO vibrational period (64 fs), before formation of a_3^* (ref. 10). However, CO is probably not yet bound to CuB during the first few hundred femtoseconds and may still interact strongly with haem a_3 in the a_3^* state. The absorption of this state, at the red side of the deliganded ground state¹⁴, is weak¹⁰, precluding the observation of clear oscillations associated with its decay. But during the first few hundred femtoseconds, the kinetics are non-exponential around 460 nm (results not shown), providing a likely explanation for the gradual phase change observed in the extracted oscillatory signal above 445 nm.

Our analysis demonstrates that the deliganded ground-state population appears in a non-smooth, stepwise manner. A more quantitative determination of the absolute population kinetics, in terms of the amplitude of the steps, requires the subtraction of the spectral evolution due to the haem *a* photophysics. A rough estimate based on such a subtraction (Fig. 4a) indicates that 80–

90% of the a_3 product state is populated during the first step, after half a vibrational period (350 fs). The remainder is populated in the subsequent steps. The small deviation of complete surface crossing allows us to assess the coupling motion, and it seems that the reaction is highly efficient and almost adiabatic. The scheme shown in Fig. 4b summarizes the results of our analysis. The observed $\sim \pi$ phase of the oscillations (Fig. 2) places the crossing region near the turning point of the motion. In a ballistic Landau–Zener picture of curve crossing, the probability of crossing P_{LZ} is inversely proportional to the absolute velocity of the packet in the crossing region¹⁵. Thus, the estimated high transfer efficiency is consistent with this region being closer to the turning point of the motion than to the minimum of the potential well.

The very low frequency (47 cm^{-1}) of the reaction coordinate mode indicates that it corresponds to a delocalized motion. Out-of-plane motion of the haem a_3 central iron atom (haem ‘doming’)

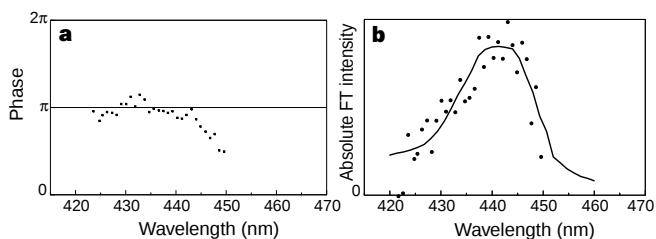


Figure 3 Wavelength dependence of the phase and integrated amplitude of the 47 cm^{-1} Fourier transform (FT) feature in CO-bound cytochrome *c* oxidase. At each individual wavelength the data were fitted by starting at $t = 50$ fs, as in Fig. 2. **a**, Phase dependence on wavelength. **b**, Integrated amplitude dependence on wavelength (circles). The integrated amplitude was determined by integrating the FT intensity of the residuals (in ΔA) over the 31 – 62 cm^{-1} range, and subtracting a noise background in the range above 400 cm^{-1} (beyond the instrument response). Solid line in **b** represents the absorption spectrum of reduced unliganded haem a_3 redrawn from ref. 11, vertically scaled to match the FT intensities.

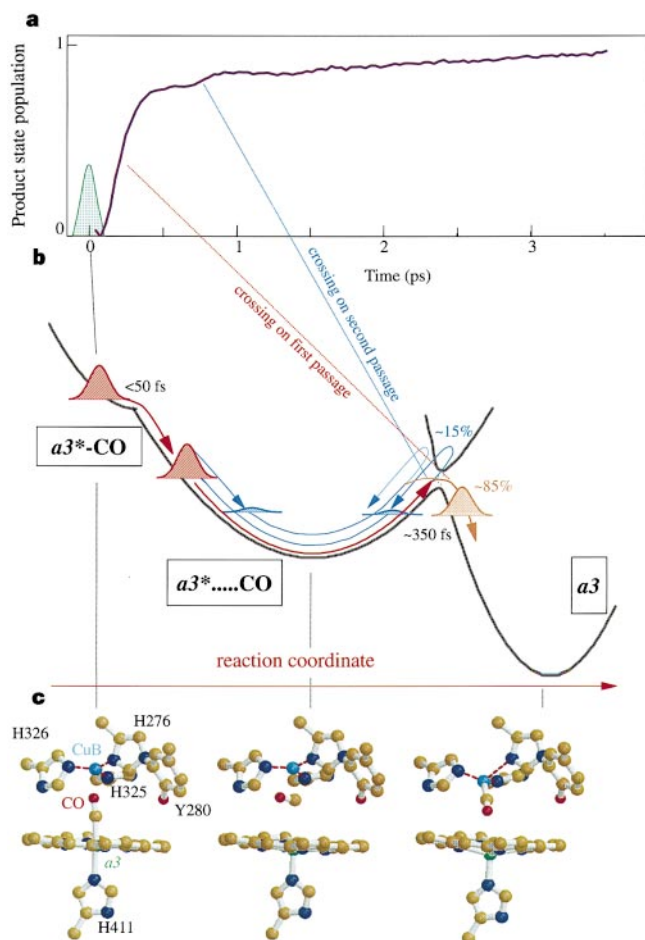


Figure 4 Kinetics and proposed mechanism of coherent population transfer. **a**, Estimate for the population of the ground-state unliganded haem a_3 . The green dot-filled curve represents the instrument-response function. **b**, Simplified scheme of the initial reaction dynamics along the reaction coordinate. Upon formation of the excited state a_3^* in less than 50 fs, a wave packet progresses along the reaction coordinate. The crossing zone to the ground state is reached after half a period (350 fs). On the first passage, 80–90% of the population decays to the ground state, and 10–20% awaits later passages. **c**, Proposed corresponding dynamics of the haem a_3 CuB site, based on molecular-dynamics energy minimizations.

should contribute strongly to structural rearrangement of the haem upon breaking of the Fe–CO bond and should occur within 1 ps, as in haemoglobin¹⁶. Furthermore, normal-mode calculations of five-coordinate haem¹⁷ and results from myoglobin¹⁸ place its frequency at $\sim 50 \text{ cm}^{-1}$, so it is likely that haem doming is involved in the observed 47 cm^{-1} reaction mode. The surface crossing occurs after half a period, when the iron ion is most displaced with respect to the haem plane.

We emphasize that the surrounding region of the haem can also be involved in this motion: in particular, haem a_3 strongly interacts with the nearby CuB centre. CuB is coordinated by three histidine residues, one of which is crosslinked with a tyrosine (Tyr 280 in the *P. denitrificans* enzyme)^{19,20} that is hydrogen-bonded to haem a_3 (ref. 19). It is probable (based on our unpublished results from molecular dynamics simulation) that haem a_3 and CuB move in a concerted way during the translation–rotation (Fig. 4) transfer of CO to CuB. It has been argued that this transfer process is ballistic, taking a minimal time of $\sim 300 \text{ fs}$ (ref. 8), corresponding well with the half-period of the 47 cm^{-1} reaction-coordinate motion.

In experiments on mitochondrial cytochrome *c* oxidase, we have found that this mechanism of ligand transfer is universal for the well conserved a_3 CuB environment of aa_3 -type oxidases. However, it does not generally apply for ligand dissociation in haem proteins

(our unpublished results): in myoglobin bound to CO, for example, the spectral properties of the oscillations are more complicated and do not indicate a coherent population transfer, and the main vibrational frequency is significantly higher (78 cm^{-1} ; compare with refs 3, 8). These findings support the idea that the haem environment is involved in motion along the reaction coordinate. As well as having different haem environments, the mechanism of association of CO ligand is different in myoglobin and cytochrome *c* oxidase: in myoglobin at least two non-coordinated docking positions for CO are reached within a few hundred femtoseconds²¹, whereas a well defined binding site (CuB) is present in cytochrome *c* oxidase. The transfer of CO to CuB may also be coupled to the reaction coordinate. Elements of the proposed structural dynamics constituting the reaction-coordinate motion, based on molecular dynamics-energy minimizations, are shown in Fig. 4c.

We have taken advantage here of the fact that CO-bound cytochrome *c* oxidase can be formed in steady state, and that ligand transfer can be initiated and synchronized by photon absorption. Our results indicate that concerted reaction dynamics in the haem a_3 CuB binuclear centre may also play a role in the biological function of the enzyme. We propose that the repulsive energy potential induced by the haem in the dissociative state generates translation–rotation motions of the diatomic molecule which optimize the probability of colliding with the CuB site, a doorstep on the way into or out of the active site. Our results provide evidence for the driving of a reaction in a protein complex by coherent motions and suggest the functional importance of vibrational coherence operating on a femtosecond/picosecond timescale. The straightforward spectroscopic identification method used here, in conjunction with femtosecond X-ray diffraction methods²², will provide a framework for mapping out reaction pathways in oxidases and other photoactivatable protein systems. □

Methods

Enzyme preparation

Wild-type *P. denitrificans* (from the Collection des Bactéries de l'Institut Pasteur, CIP 71.11) was grown as described²³. Cytochrome oxidase aa_3 was purified as described²⁴ except that Q-Sepharose was replaced by Source 30Q (Pharmacia) and the final step using a chelating column was omitted. Dithionite (20 mM)-reduced unliganded and CO-liganded samples (protein concentration, $68 \mu\text{M}$) were prepared in gas-tight cells with an optical path length of 1 mm.

Femtosecond spectroscopy

Multicolour femtosecond spectroscopy²⁵ was performed at a repetition rate of 30 Hz with compressed white light continuum probe pulses and 55-fs (full width at half-maximum) pump pulses centred at 590 nm, in the α -band. In contrast to the Soret band, the Raman cross-section for the low-frequency modes is very small for excitation in the α -band of haems²⁶. Transient spectra were simultaneously measured using an optical multichannel analyser.

Data analysis

Noise in the data was reduced using singular value decomposition²⁷. Single-wavelength curves were fitted starting at 50 fs, with two exponential components (time constants of $\sim 130 \text{ fs}$ and $\sim 2.5 \text{ ps}$) and a constant. Allowing for an additional exponential component did not improve the fit. The population kinetics of the ground-state unliganded haem a_3 were estimated by subtracting the 442-nm transient absorption kinetics of the CO-liganded enzyme and the fit of the kinetics of the unliganded enzyme of Fig. 2, after normalization on the initial bleachings. This normalization is based on the observation that the contribution at 442 nm of the a_3 -CO absorption to the total aa_3 -CO absorption is weak (from ref. 11 and from the known shift of the a_3 band upon CO binding, we estimate this to be $<10\%$) and the initial bleachings should reflect predominantly unliganded haem in both cases.

Structural modelling

The models shown in Figs 1a and 4c are adapted from the *P. denitrificans* two-subunit/antibody Fv fragment-structure of ref. 20 (Brookhaven Protein Data Bank, file 1ar1). Initially, CO was bound to the haem a_3 iron, using the Fe–C bond length ($1.90 \text{ \AA}$) reported for the bovine heart cytochrome *c* oxidase–CO complex¹⁹. Subsequently, the energy of the structure was minimized, using version 24 (parameter version 22) of CHARMM²⁸. A cut-off function, acting between 10 and $13 \text{ \AA}$, gradually eliminated non-covalent interactions and a relative dielectric constant of 1 was used for the entire structure. The minimization

procedure consisted of an initial 20 steps of the 'steepest descent' algorithm, followed by 200 steps of the 'adopted basis Newton-Raphson' (ABNR) algorithm. In the minimized structure (Figs 1a and 4c (left)), the iron ion lies in the plane of the haem. Subsequently, the Fe-C bond was broken and the structure was minimized using the ABNR algorithm. Figure 4c (middle) represents an intermediate step, where the haem is partially domed and the CO molecule is closest to CuB, in the transition zone between iron a_3 bound and CuB bound. Finally, CO was bound to CuB and the structure minimized using 50 steps of the ABNR algorithm (Fig. 4c, right). In this structure, the iron ion lies out of the haem plane (0.63 Å) and the copper-bound CO molecule makes an angle of 55° with the normal of the haem a_3 plane, consistent with the angle of 55° inferred from polarized transient infrared measurements⁸. The programs MOLSCRIPT²⁹ and RASTER3D³⁰ were used to prepare the figures.

Received 23 April; accepted 30 June 1999.

- Vos, M. H., Rappaport, F., Lambry, J.-C., Breton, J. & Martin, J.-L. Visualization of coherent nuclear motion in a membrane protein by femtosecond spectroscopy. *Nature* **363**, 320–325 (1993).
- Wang, Q., Schoenlein, R. W., Peteanu, L. A., Mathies, R. A. & Shank, C. V. Vibrationally coherent photochemistry in the femtosecond primary event of vision. *Science* **266**, 422–424 (1994).
- Zhu, L., Sage, J. T. & Champion, P. M. Observation of coherent reaction dynamics in heme proteins. *Science* **266**, 629–632 (1994).
- Vos, M. H. *et al.* Direct observation of vibrational coherence in bacterial reaction centers using femtosecond absorption spectroscopy. *Proc. Natl Acad. Sci. USA* **88**, 8885–8889 (1991).
- Babcock, G. T. & Wikström, M. Oxygen activation and the conservation of energy in cell respiration. *Nature* **356**, 301–309 (1992).
- Lemon, D. D., Calhoun, M. W., Gennis, R. B. & Woodruff, W. H. The gateway to the active site of heme-copper oxidases. *Biochemistry* **32**, 11953–11956 (1993).
- Verkhovsky, M. I., Morgan, J. E. & Wikström, M. Oxygen binding and activation: early steps in the reaction of oxygen with cytochrome *c* oxidase. *Biochemistry* **33**, 3079–3086 (1994).
- Dyer, R. B., Peterson, K. A., Stoutland, P. O. & Woodruff, W. H. Picosecond infrared study of the photodynamics of carbonmonoxide-cytochrome *c* oxidase. *Biochemistry* **33**, 500–507 (1994).
- Schulvis, J. P. M., Deinum, G., Varotsis, C. A., Ferguson-Miller, S. & Babcock, G. T. Low-power picosecond resonance Raman evidence for histidine ligation to heme a_3 after photodissociation of CO from cytochrome *c* oxidase. *J. Am. Chem. Soc.* **119**, 8409–8416 (1997).
- Stoutland, P. O., Lambry, J.-C., Martin, J.-L. & Woodruff, W. H. Femtosecond dynamics of reduced cytochrome *c* oxidase and its CO derivative. *J. Phys. Chem.* **95**, 6406–6408 (1991).
- Vanneste, W. H. The stoichiometry and absorption spectra of components *a* and a_3 in cytochrome *c* oxidase. *Biochemistry* **5**, 838–848 (1966).
- Vos, M. H., Jones, M. R. & Martin, J.-L. Vibrational coherence in bacterial reaction centers: spectroscopic characterisation of motions active during primary electron transfer. *Chem. Phys.* **233**, 179–190 (1998).
- Georgiadis, K. E., Jhon, N.-I. & Einarsson, Ö. Time-resolved optical absorption studies of intramolecular electron transfer in cytochrome *c* oxidase. *Biochemistry* **33**, 9245–9256 (1994).
- Martin, J.-L. *et al.* in *Ultrafast Phenomena IV* (eds Auston, D. H. & Eisinger, K. B.) 447–451 (Springer, Berlin, 1984).
- Bagchi, B. & Fleming, G. R. Dynamics of activationless reactions in solution. *J. Phys. Chem.* **94**, 9–20 (1990).
- Franzen, S., Lambry, J.-C., Bohn, B., Poyart, C. & Martin, J.-L. Direct evidence for the role of haem doming as the primary event in the cooperative transition of haemoglobin. *Nature Struct. Biol.* **1**, 230–233 (1994).
- Li, X.-Y. & Zgierski, M. Z. Iron motion in a five-coordinated heme model. *Chem. Phys. Lett.* **188**, 16–20 (1992).
- Champion, P. M. *et al.* in *Proceedings of XVth International Conference on Raman Spectroscopy* (ed. Heyns, P. M.) 73–76 (Wiley, Chichester, 1998).
- Yoshikawa, S. *et al.* Redox-coupled structure changes in bovine heart cytochrome *c* oxidase. *Science* **280**, 1723–1729 (1998).
- Ostermeier, C., Harrenga, A., Ermiler, U. & Michel, H. Structure at 2.7 Å resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody F₁ fragment. *Proc. Natl Acad. Sci. USA* **94**, 10547–10553 (1997).
- Lim, M., Jackson, T. A. & Anfinsen, P. A. Ultrafast rotation and trapping of carbon monoxide dissociated from myoglobin. *Nature Struct. Biol.* **4**, 209–214 (1997).
- Rischel, C. *et al.* Femtosecond time-resolved X-ray diffraction from laser-heated organic films. *Nature* **390**, 490–492 (1997).
- Ludwig, B. Cytochrome *c* oxidase from *Paracoccus denitrificans*. *Meth. Enzymol.* **126**, 153–159 (1986).
- Haltia, T., Semo, N., Arrondo, J. L. R., Goñi, F. M. & Freire, E. Thermodynamic and structural stability of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Biochemistry* **33**, 9731–9740 (1994).
- Martin, J.-L. & Vos, M. H. Femtosecond measurements of geminate recombination in heme proteins. *Methods Enzymol.* **232**, 416–430 (1994).
- Asher, S. Resonance Raman spectroscopy of hemoglobin. *Methods Enzymol.* **76**, 371–413 (1981).
- Rischel, C. *et al.* Low-frequency vibrational modes in proteins: changes induced by point-mutations in the protein-cofactor matrix of bacterial reaction centers. *Proc. Natl Acad. Sci. USA* **95**, 12306–12311 (1998).
- Brooks, B. R., Brucoleri, R. E., Olafson, B. D., Swaminathan, S. & Karplus, M. CHARMM: a program for macromolecular energy, minimisation, and dynamics calculations. *J. Comput. Chem.* **4**, 187–212 (1983).
- Kraulis, P. J. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950 (1991).
- Merritt, E. A. & Bacon, D. J. Raster3D—photorealistic molecular graphics. *Methods Enzymol.* **277**, 505–524 (1997).

Acknowledgements

U.L. and M.H.V. were supported by CNRS.

Correspondence and requests for materials should be addressed to M.H.V. (e-mail: vos@enst.fr).

Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor

Christian Wiesmann*, Mark H. Ultsch*, Steven H. Bass†‡ & Abraham M. de Vos*

Departments of* Protein Engineering and † Molecular Biology, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, USA

Nerve growth factor (NGF) is involved in a variety of processes involving signalling, such as cell differentiation and survival, growth cessation and apoptosis of neurons¹. These events are mediated by NGF as a result of binding to its two cell-surface receptors, TrkA and p75 (ref. 2). TrkA is a receptor with tyrosine kinase activity that forms a high-affinity binding site for NGF³. Of the five domains comprising its extracellular portion, the immunoglobulin-like domain proximal to the membrane (TrkA-d5 domain) is necessary and sufficient for NGF binding⁴. Here we present the crystal structure of human NGF in complex with human TrkA-d5 at 2.2 Å resolution. The ligand-receptor interface consists of two patches of similar size. One patch involves the central β-sheet that forms the core of the homodimeric NGF molecule and the loops at the carboxy-terminal pole of TrkA-d5. The second patch comprises the amino-terminal residues of NGF, which adopt a helical conformation upon complex formation, packing against the 'ABED' sheet of TrkA-d5. The structure is consistent with results from mutagenesis experiments for all neurotrophins, and indicates that the first patch may constitute a conserved binding motif for all family members, whereas the second patch is specific for the interaction between NGF and TrkA.

Besides NGF, the neurotrophin family⁵ includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 and neurotrophin-6 (ref. 6), all of which share a pair-wise

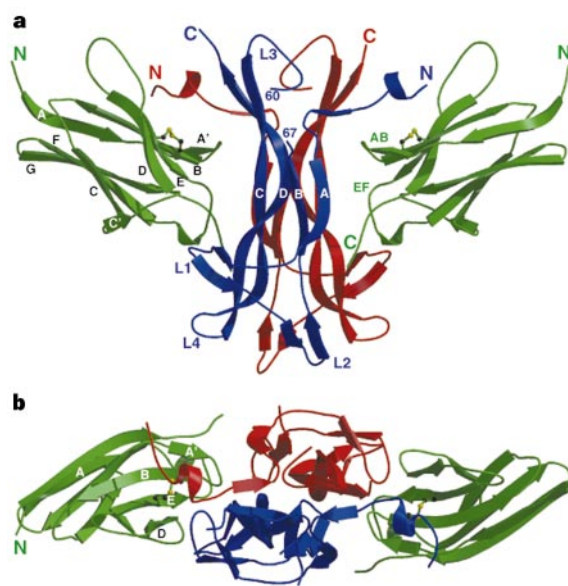


Figure 1 Overall structure of the complex. **a**, **b**, Two orientations related by a rotation of 90° about the horizontal axis. The NGF monomers are red and blue; TrkA-d5 is green. The termini, some relevant loops and the secondary structure elements are labelled. In **a**, loops L2 and L4 of NGF and the C-terminus of TrkA-d5 (residue 382) point towards the membrane.

sequence identity of about 50%. The extracellular portions of the neurotrophin receptors, TrkA, TrkB and TrkC, have a common architecture comprising a leucine-rich domain (domain 2) sandwiched between two cysteine-rich clusters, followed by two immunoglobulin(Ig)-like domains (domains 4 and 5)⁷. Domains 4 and 5 of TrkA have been suggested to carry the NGF-binding determinants^{4,8}; domain 5 by itself binds to NGF with an affinity similar to that of the native receptor⁴ (D. Dawbarn, personal communication), is sufficient for neurotrophin-dependent activation of the intracellular kinase domain⁴ and can antagonize NGF *in vitro* and *in vivo* (D. Dawbarn, personal communication).

For our crystallographic studies, we used a construct consisting of domains 4 and 5 together with the linker connecting domain 5 to the transmembrane segment. However, over the long time it took for crystals to appear, the TrkA protein was partially proteolysed, and the resulting crystals contain a complex consisting of one symmetrical NGF homodimer and two copies of only domain 5 of TrkA (residues 280–380) together with the first three linker residues (381–383). The overall shape of this complex resembles a bat with a length of 60 Å and a wing span of 95 Å, its body formed by the NGF dimer and the wings represented by the TrkA-d5 molecules (Fig. 1). The non-crystallographically-related halves of the complex are very similar to each other. With a root-mean-square (r.m.s.) difference of only 0.24 Å for 101 common Cα positions, the TrkA-d5 molecules are virtually identical, whereas for NGF small differences in the flexible loops around residues 46 and 95 result in a slightly larger r.m.s. deviation of about 0.7 Å between the two monomers (108 Cα pairs).

TrkA-d5 folds into an Ig-like domain and consists of a β-sandwich formed by two four-stranded sheets, one comprising strands A, B, E and D, and the other strands C', C, F and G. In the crystal structure of TrkA-d5 in its unbound state⁹, this domain was misfolded into artificial dimers, with strand A of one molecule replaced by the same strand of a symmetry-related molecule. The conformation of the correctly folded domain was modelled from this dimer by changing the position of one residue in the AB loop, with the assumption that no other changes were introduced by the observed strand swapping. Superposition of this modelled TrkA-d5 structure in its unbound state onto the structure of TrkA-d5 taken from the complex with NGF results in an r.m.s. difference of only about 0.5 Å for 98 Cα positions. Therefore, we tentatively conclude that ligand binding does not induce any conformational changes in TrkA-d5.

In contrast, a comparison with the structure of unbound murine NGF¹⁰ reveals three regions of significant changes in the ligand, some induced by receptor binding. The core of the NGF monomer is formed by a pair of two-stranded, twisted β-sheets (part of the 'waist' of the bat; Fig. 1) with a cysteine-knot motif and a reverse turn (L3) at one end and three β-hairpin loops (L1, L2 and L4) at the other. Two monomers assemble in a parallel manner to form the physiological dimer. Overall, the structure of bound human NGF is very similar to the previously reported structures, as reflected in r.m.s. deviations of 0.8–1.2 Å for about 100 common Cα-positions. Of the local differences, one is situated in the interface between the two NGF monomers, where the side chain of Trp 99 adopts a different conformation in the complex; it is unlikely that this difference is a result of complex formation. The second difference involves residues 61–66. These amino acids are solvent exposed but well defined in the murine NGF structures, and are disordered in our structure. We believe that this change may be the result of sequence differences between the human and murine proteins (Fig. 2a). The final and most prominent conformational change concerns the N-terminal residues 2–9. This segment is important for receptor binding¹¹, but was disordered in all previously reported NGF structures¹⁰. In contrast, these residues are very well defined in the receptor complex and form an important part of the interface with TrkA-d5 (see below).

Overall, about 2,220 Å² of solvent-accessible surface is buried between NGF and each copy of TrkA-d5. Of the 1,120 Å² buried on the surface of each TrkA-d5 molecule, about 70% is due to residues from one NGF monomer and the remaining 30% to the other. The interface can be divided into two patches (Fig. 3a). On TrkA-d5, the larger of these patches covers most of one pole of the domain, accounting for about 55% of the total buried surface and involving residues from the AB, C'D and EF loops, together with the C-terminus. Note that the residues at the tip of the AB loop, which were responsible for the strand swapping in the structure of unbound TrkA-d5 (ref. 9), are in intimate contact with NGF. On NGF, the first patch involves one of the β-hairpin loops (L1) and residues from the four β-strands that form the waist of the molecule. One of these residues is Arg103, which is strictly conserved in all neurotrophins (Fig. 2a) and is the most important binding determinant for the interaction between NT-3 and TrkC¹². In the NGF–TrkA-d5 complex, its side chain protrudes from the central β-sheet of NGF to stack against the phenyl group of Phe 327 of TrkA, simultaneously forming a hydrogen bond with the carbo-

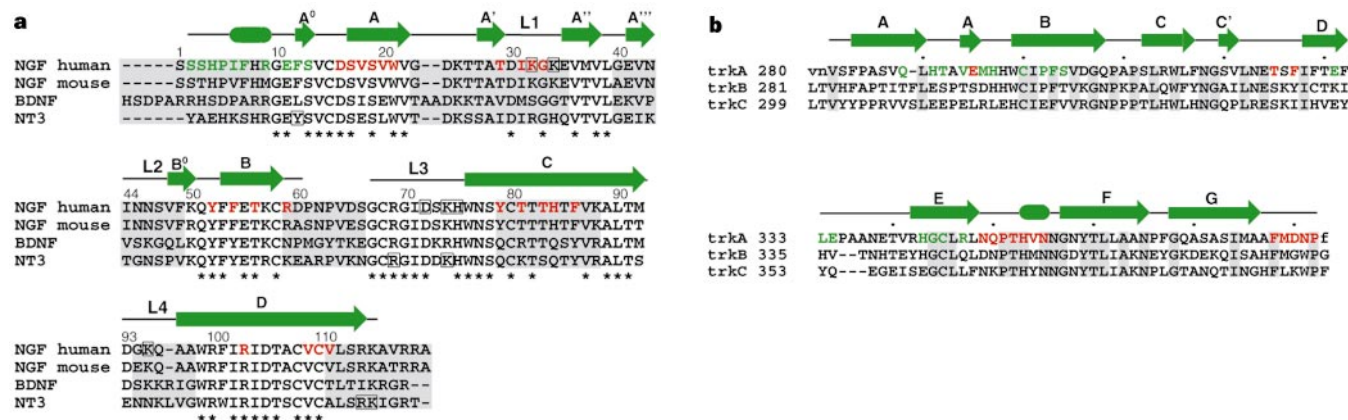


Figure 2 Sequence alignments. **a**, The neurotrophins. The numbering and secondary structure elements above the sequence refer to mature human NGF. Conserved residues are marked with a star, and regions with low sequence homology as defined in ref. 16 are shaded. Residues in contact with TrkA-d5 are red (common patch) and green (specificity patch), and amino acids important for p75 binding are boxed. **b**, Domain 5 of the Trk

receptors. Secondary structure elements above the sequences pertain to TrkA-d5. Conserved residues are shaded. TrkA residues in the common patch are red and residues in the specificity patch are green. Residues shown in lower case are present in our crystals but disordered. Residues 381–383 are the start of the linker region to the transmembrane segment.

nyl oxygen of Asn 349 (Fig. 3b). In this patch, 14 of the 23 NGF residues are occupied by a homologous amino acid in BDNF and NT-3; similarly, 8 out of 15 TrkA residues are conserved in TrkB and TrkC (Fig. 2b). We therefore speculate that, in this patch, interactions similar to those observed here are conserved among all neurotrophin–receptor complexes.

The second patch is formed by N-terminal residues 2–13 of NGF, packing against the solvent-exposed face of the ABED sheet of TrkA-d5 (Fig. 1). Here, the amino acids in the interface share little

sequence conservation, both among the ligands (2 homologous residues out of 10 total) and among the receptors (4 of 16). The N-terminal residues of the neurotrophins are important for specificity; indeed, a hybrid of NT-3 with the N terminus replaced with that of NGF has high affinity not only for TrkC but also for TrkA¹². The role of the N terminus of NGF becomes obvious from the structure of the complex (Fig. 3c). Residues 6–9 adopt a helical conformation; with their side chains almost completely buried in the interface, His4 and Ile6 appear to be the most important receptor-binding

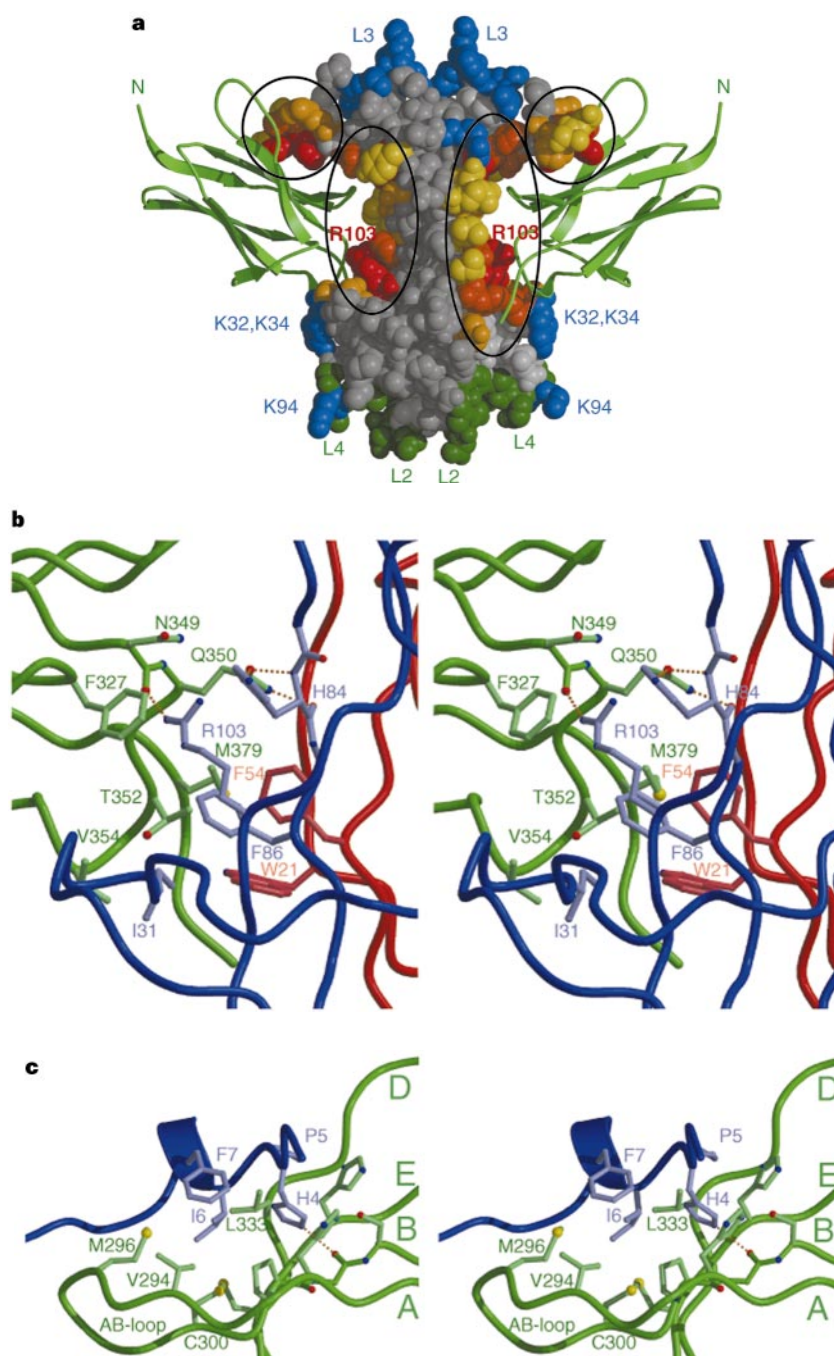


Figure 3 The interface between NGF and TrkA-d5. **a**, The binding epitopes of NGF to its receptors TrkA and p75. NGF is shown as a space-filling model, and TrkA-d5 in ribbon rendering. The NGF residues in the interface with TrkA-d5 are colour-coded in different shades of red, reflecting the percentage of accessible surface buried in the interface (dark red, 75–100%; orange, 50–75%; light orange, 25–50%; yellow, 0–25%). The conserved and specificity patches in the interface are outlined (circles: the two

symmetrical specificity patches; ovals: the two symmetrical common patches), and Arg 103 (in red) on each NGF monomer is labelled. Loops L2 and L4, which are proposed to interact with the linker between TrkA-d5 and the membrane, are dark green. Residues important for binding to p75 (refs 12, 23) are blue. **b**, Stereo view showing the environment of Arg 103 of NGF in the conserved portion of the interface. **c**, Stereo view showing interactions between the N terminus of NGF and the specificity patch of TrkA-d5.

determinants in this patch. Ile 6 is embedded into a hydrophobic canyon on the surface of TrkA-d5. The floor of this canyon is formed by the unusual disulphide bridge on the surface of TrkA-d5 (ref. 9), with the side chains of Val 294 and Leu 333 lining the walls. Pro 5 and Phe 7 of NGF also contribute to the hydrophobic interactions in this region, while His 4 and Glu 11 form hydrogen bonds to Ser 304 and Arg 347 of TrkA-d5. Mutation to alanine of the residues forming this canyon significantly decreases the binding affinity of TrkA for NGF, but not that of TrkC for NT-3 (ref. 13). We conclude that this second patch is responsible for specificity among the neurotrophins.

As our crystallized complex includes only the fifth domain and a few of the following linker residues of TrkA, potential interactions between NGF and other parts of the receptor are less clear. The last visible residue of our TrkA construct (residue 382) ends in the vicinity of and projects towards two loops on the membrane-facing side of NGF (Figs 1, 3a). These loops, L2 and L4, represent two of seven distinct regions with higher than average sequence diversity among the neurotrophins (Fig. 2a). Swapping experiments between NGF and BDNF of these seven regions showed that L2 and L4 are important for the biological activity of NGF¹⁴. Furthermore, substitution of five NT-3 residues in loops L2 and L4 with those of NGF introduced NGF-like activity while maintaining NT-3 activity¹⁵. We speculate that these loops may interact with some of the C-terminal 40 residues of the linker region of TrkA, which are not present in our crystals. This is further supported by experiments indicating that mutation of some of these linker residues affects binding to NGF¹³.

The crystal structure of the NGF-TrkA-d5 complex is entirely consistent with mutagenesis studies on not only NGF, but also BDNF and NT-3 (refs 16–18). This contradicts the current belief, based on mutagenesis studies in which segments were swapped between homologous ligands, that these neurotrophins have structurally distinct mechanisms of receptor binding and activation¹¹. The explanation for this discrepancy is that, although swapping experiments can reveal binding determinants that are specific for some of the ligands, areas of common importance would be missed. Our crystal structure taken together with these data indicates that all neurotrophins bind to their respective Trk receptors in the same, conserved manner.

The structure of the complex does not provide clear insight into the role of domains 1–4 of TrkA, except that they are unlikely to be directly involved in ligand binding. Sequence alignments indicate that domains 4 and 5 may be connected through a very short linker; given the position of the N terminus of TrkA-d5, it seems impossible that domain 4 could be positioned near the ligand. It has been suggested that domain 2 binds to NGF with nanomolar affinity¹⁹ and that it is important for binding to BDNF and NT-4/5 (ref. 20). However, it is not obvious from the present structure how direct contact could be achieved. On the contrary, the observation that all the neurotrophin residues important for binding and/or specificity are in the interface to domain 5 (or the immediately following linker residues) strongly supports the notion that the binding site of the Trk receptors is almost entirely contained within our structure, the only exception being the linker residues that are missing from our crystals. This conclusion is consistent with binding studies on TrkA (D. Dawbarn, personal communication) and deletion studies on TrkA, TrkB and TrkC⁴ showing that domain 5 binds to the neurotrophin ligands with near wild-type affinity.

In contrast to the Trk receptors, which are specific for their ligands, the p75 receptor binds to all neurotrophins with similar, but lower, affinity²¹. p75 is a member of the tumour necrosis factor receptor family and promotes apoptosis when NGF binds in the absence of TrkA; in the presence of TrkA, high-affinity binding sites are present on the cell surface that promote survival at low NGF concentrations. Two binding epitopes for p75 have been identified on the surface of NGF (Fig. 3a). One patch involves mostly positively charged residues in loops L1 and L4 at the surface proximal to the membrane²², whereas the second patch contains

hydrophilic residues from the highly conserved loop L3 and the C terminus^{12,23}. In our complex, the side chains of only two of these residues have minor contacts to TrkA-d5, indicating that the neurotrophins could bind to p75 and Trk simultaneously.

This structure and the structure of the complex between vascular endothelial growth factor (VEGF) and domain 2 of Flt-1 (ref. 24) are two examples of structurally related ligands in complex with the binding domain of their respective receptor tyrosine kinases. Both ligands belong to a family of dimeric, cysteine-knot containing growth factors with a similar elongated monomer architecture²⁵, and the ligand-binding domains of the receptors are both Ig-like domains of the I-set variety. Despite these similarities, the differences in dimerization mode of the ligands result in entirely different binding modes. The parallel NGF dimer contains binding sites disposed symmetrically along the thin end of the molecule, placing the two TrkA-d5 molecules only 20 Å apart, whereas the widely separated binding sites at the poles of the antiparallel VEGF dimer result in a 65 Å distance between the two bound receptors. For the receptors, the binding sites are also located in completely different regions of the structure. TrkA-d5 utilises loops at the pole of the domains together with a patch on the ABED sheet, whereas domain 2 of Flt-1 interacts with VEGF through its A'GFC surface. The unexpected result is that, despite the similarities in the building blocks, each signalling complex is assembled in a unique and unpredictable manner. Structures of additional ligand–receptor complexes within the family are needed to reveal whether any common themes might exist. □

Methods

Expression and crystallization

Escherichia coli cells were transfected with the JAL172 vector including residues 192–408 of the human TrkA receptor linked to an N-terminal STII signal sequence under the alkaline phosphatase promoter. Inclusion bodies were solubilized in 8 M urea, 50 mM TRIS, pH 8.0 and 2.5 mM EDTA, and a cocktail of protease inhibitors was added. After size-exclusion chromatography (S200), the protein was dialysed against 0.5 M TRIS, pH 8.5, concentrated and further purified by exclusion (S200) and anion-exchange chromatography. Complex was formed by adding a slight molar excess to recombinant human NGF (Genentech) and purified using size-exclusion chromatography (S75). For crystallization by the hanging drop method, the drops consisted of 2 µl protein solution (10 mg ml⁻¹ complex in 0.1 M NaCl, 0.1 M bicine, pH 8.5) mixed with 2 µl of reservoir, using a low ionic strength screen (Hampton). Small crystals appeared after three months; macro seeding into a 1:1 mixture of protein solution and crystallization buffer (24% PEG 3350, 0.1 M citric acid, pH 5.0) resulted in crystals with dimensions of 100 × 100 × 250 µm after six months. Mass spectrometry analysis and N-terminal sequencing of protein obtained from dissolved crystals showed that the receptor had been proteolysed to residues 280–383.

Data collection, structure determination and refinement

A crystal was flash-frozen after dipping in 20% MPD, and data were collected at beamline 9-1 of the Stanford Synchrotron Radiation Laboratory and processed with HKL²⁶ ($R_{\text{merge}} = 12.7\%$, 96% complete to 3.2 Å). The crystals belong to space group $P2_1$ with cell parameters of $a = 58.7$ Å, $b = 56.1$ Å, $c = 79.2$ Å and $\beta = 109.3^\circ$, and contain one complex in the asymmetric unit. Search models for molecular replacement using program AMoRe²⁷ were based on the structures of unbound TrkA-d5 (ref. 9) and murine NGF (Protein Data Bank accession number IBTG). A search with the NGF dimer gave a clear solution; fixing this solution allowed the expected two copies of TrkA-d5 to be placed. After rigid body refinement, the correlation coefficient was 38.4% (R -value 47.4%) using all measured reflections between 12 and 4 Å resolution. Alternative cycles of model building with program O²⁸ and positional refinement using programs REFMAC²⁷ and X-PLOR²⁹ resulted in an R -value of 27.8% ($R_{\text{free}} = 33.4\%$ for 10% of the reflections not used in refinement). A crystal stabilized in artificial mother liquor with added MPD was flash-frozen and used to collect a 2.2 Å data set at beam line 5.2 of the Advanced Light Source, Berkeley. The cell parameters were $a = 58.2$ Å, $b = 53.7$ Å, $c = 77.1$ Å and $\beta = 107.4^\circ$, indicating that the unit cell volume had shrunk by 6.6% compared to the first crystal. The data set was 99.8% complete between 20 and 2.2 Å resolution, comprising 23,361 unique reflections with an R_{merge} of 7.1% (35.5% in the highest resolution shell). The original set of free R -value reflections was extended to 2.2 Å, and further refinement using non-crystallographic symmetry restraints, inclusion of water molecules and application of a solvent mask resulted in a final R -value of 18.9% ($R_{\text{free}} = 26.6\%$). The model consists of residues 2–60 and 67–116 of each NGF monomer, residue 117 of one of the NGF monomers, residues 282–382 of each copy of TrkA-d5, and 209 water molecules (3,492 non-hydrogen atoms). Of all non-proline/non-glycine residues, 87% are in the 'most favoured'³⁰ regions of the Ramachandran plot and none are in the 'disallowed' regions. Two segments of NGF (residues 45–48 and 93–95) have poorly defined electron density and contain the only three residues located in the 'generously allowed' regions. The r.m.s. differences from dictionary bond lengths and angles are 0.010 Å and 1.9°, respectively, and

the r.m.s. difference in B-factors of bonded atoms is 4.1 Å².

Received 21 January; accepted 5 July 1999.

- Snider, W. D. Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* **77**, 627–638 (1994).
- Chao, M. *et al.* Neurotrophin receptors: mediators of life and death. *Brain Res. Rev.* **26**, 295–301 (1998).
- Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V. & Parada, L. F. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* **252**, 554–558 (1991).
- Urfür, R. *et al.* An immunoglobulin-like domain determines the specificity of neurotrophin receptors. *EMBO J.* **14**, 2795–2805 (1995).
- Chao, M. V. Neurotrophin receptors: a window into neuronal differentiation. *Neuron* **9**, 583–593 (1992).
- Götz, R. *et al.* Neurotrophin-6 is a new member of the nerve growth factor family. *Nature* **372**, 366–369 (1994).
- Schneider, R. & Schweiger, M. A novel modular mosaic of cell adhesion motifs in the extracellular domains of the neurogenic trk and trkB tyrosine kinase receptors. *Oncogene* **6**, 1807–1811 (1991).
- Peréz, P., Coll, P. M., Hempstead, B. L., Martin-Zanca, D. & Chao, M. V. NGF binding to the trk tyrosine kinase receptor requires the extracellular immunoglobulin-like domains. *Mol. Cell. Neurosci.* **6**, 97–105 (1995).
- Ullsch, M. *et al.* Crystal structures of the neurotrophin-binding domain of TrkA, TrkB and TrkC. *J. Mol. Biol.* **290**, 149–159 (1999).
- McDonald, N. Q. *et al.* New protein fold revealed by a 2.3-Å resolution crystal structure of nerve growth factor. *Nature* **354**, 411–414 (1991).
- McInnes, C. & Sykes, B. D. Growth factor receptors: structure, mechanism, and drug discovery. *Biopolymers* **43**, 339–366 (1997).
- Urfür, R. *et al.* The binding epitopes of neurotrophin-3 to its receptors trkC and gp75 and the design of a multifunctional human neurotrophin. *EMBO J.* **13**, 5896–5909 (1994).
- Urfür, R. *et al.* High resolution mapping of the binding site of TrkA for nerve growth factor and TrkC for neurotrophin-3 on the second immunoglobulin-like domain of the Trk receptors. *J. Biol. Chem.* **273**, 5829–5840 (1998).
- Ibáñez, C. F., Ebendal, T. & Persson, H. Chimeric molecules with multiple neurotrophic activities reveal structural elements determining the specificities of NGF and BDNF. *EMBO J.* **10**, 2105–2110 (1991).
- Kullander, K. & Ebendal, T. Neurotrophin-3 acquires NGF-like activity after exchange to five NGF amino acid residues: molecular analysis of the sites in NGF mediating the specific interaction with the NGF high affinity receptor. *J. Neurosci. Res.* **39**, 195–210 (1994).
- Ibáñez, C. F., Ilag, L. L., Murray-Rust, J. & Persson, H. An extended surface of binding to Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin. *EMBO J.* **12**, 2281–2293 (1993).
- O'Leary, P. D. & Hughes, R. A. Structure-activity relationships of conformationally constrained peptide analogues of loop 2 of brain-derived neurotrophic factor. *J. Neurochem.* **70**, 1712–1721 (1998).
- Urfür, R., Tsoulfas, P., O'Connell, L. & Presta, L. G. Specificity determinants in neurotrophin-3 and design of nerve growth factor-based trkC agonists by changing central beta-strand bundle residues to their neurotrophin-3 analogs. *Biochemistry* **36**, 4775–4781 (1997).
- Windisch, J. M., Marksteiner, R. & Schneider, R. Nerve growth factor binding site on TrkA mapped to a single 24-amino acid leucine-rich motif. *J. Biol. Chem.* **270**, 28133–28138 (1995).
- Windisch, J. M., Marksteiner, R., Lang, M. E., Auer, B. & Schneider, R. Brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 bind to a single leucine-rich motif of TrkB. *Biochemistry* **34**, 11256–11263 (1995).
- Dechant, G., Tsoulfas, P., Parada, L. F. & Barde, Y. A. The neurotrophin receptor p75 binds neurotrophin-3 on sympathetic neurons with high affinity and specificity. *J. Neurosci.* **17**, 5271–5287 (1997).
- Ibáñez, C. F. *et al.* Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. *Cell* **69**, 329–341 (1992).
- Ryden, M. & Ibáñez, C. F. A second determinant of binding to the p75 neurotrophin receptor revealed by alanine-scanning mutagenesis of a conserved loop in nerve growth factor. *J. Biol. Chem.* **272**, 33085–33091 (1997).
- Wiesmann, C. *et al.* Crystal structure at 1.7 Å resolution of VEGF in complex with domain 2 of the Flt-1 receptor. *Cell* **91**, 695–704 (1997).
- Sun, P. D. & Davies, D. R. The cystine-knot growth-factor superfamily. *Annu. Rev. Biophys. Biomol. Struct.* **24**, 269–291 (1995).
- Otwiniński, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307–326 (1997).
- CCP4. Programs for protein crystallography. *Acta Crystallogr. D* **50**, 760–763 (1994).
- Jones, T. A., Zhou, J.-Y., Cowan, S. W. & Kjeldgaard, M. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A* **47**, 110–119 (1991).
- Brünger, A. T., Kuriyan, J. & Karplus, M. Crystallographic R factor refinement by molecular dynamics. *Science* **235**, 458–460 (1987).
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. Procheck: a program to check to the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291 (1993).

Acknowledgements

We thank the following colleagues at Genentech: E. Martin and G. Burton for purified NGF; D. Reilly for fermentation runs; W. Henzel for N-terminal sequencing; J. Bourell for mass spectrometry analysis; H. Christinger for help with data collection; and L. Presta and D. Shelton for helpful discussions. We also thank the staff at SSRL, beam line 9-1, and ALS, beam line 5.2; and D. Dawbarn for sharing data before publication.

Correspondence and requests for materials should be directed to A.M.D.V. (e-mail: devos@gene.com). The coordinates have been deposited with the Protein Data Bank for immediate release (accession number 1www).

Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors

Michael S. Finnin*, Jill R. Donigian†, Alona Cohen†, Victoria M. Richon‡, Richard A. Rifkind‡, Paul A. Marks‡, Ronald Breslow§ & Nikola P. Pavletich*

† Cellular Biochemistry and Biophysics Program and * Howard Hughes Medical Institute, ‡ Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA

§ Department of Chemistry, Columbia University, New York, New York 10027, USA

Histone deacetylases (HDACs) mediate changes in nucleosome conformation and are important in the regulation of gene expression¹. HDACs are involved in cell-cycle progression and differentiation, and their deregulation is associated with several cancers^{2,3}. HDAC inhibitors, such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), have anti-tumour effects, as they can inhibit cell growth^{4–6}, induce terminal differentiation^{4,5} and prevent the formation of tumours in mice models^{7,8}, and they are effective in the treatment of promyelocytic leukemia³. Here we describe the structure of the histone deacetylase catalytic core, as revealed by the crystal structure of a homologue from the hyperthermophilic bacterium *Aquifex aeolicus*, that shares 35.2% identity with human HDAC1 over 375 residues, deacetylates histones *in vitro* and is inhibited by TSA and SAHA. The deacetylase, deacetylase-TSA and deacetylase-SAHA structures reveal an active site consisting of a tubular pocket, a zinc-binding site and two Asp-His charge-relay systems, and establish the mechanism of HDAC inhibition. The residues that make up the active site and contact the inhibitors are conserved across the HDAC family. These structures also suggest a mechanism for the deacetylation reaction and provide a framework for the further development of HDAC inhibitors as anti-tumour agents.

HDACs catalyse the removal of acetyl groups for the ε-amino groups of lysine residues clustered near the amino terminus of nucleosomal histones, and this process is associated with transcriptional repression⁹. The deregulation of HDAC recruitment to promoters appears to be one of the mechanisms by which these enzymes contribute to tumorigenesis. For example, in acute promyelocytic leukemia (APL), chromosomal translocations fuse the retinoic acid receptor-α (RARα) to either the promyelocytic leukaemia zinc finger (PLZF) or the promyelocytic leukaemia protein (PML), which recruit a co-repressor and, in turn, HDACs³. There are two related classes of eukaryotic HDACs (Class I and II; refs 10–12) and they share a ~390-amino-acid region of homology—the deacetylase core. The deacetylase core belongs to a superfamily¹³ of genes that includes the *A. aeolicus* HDAC homologue (35.2% identity with HDAC1), the prokaryotic acetoin utilization proteins (AcuC; 28.1% identity with HDAC1) and the prokaryotic acetyl-polyamine amidohydrolases (APAH; 15.0% identity with HDAC1).

The function of the *A. aeolicus* HDAC homologue is unknown, but it can deacetylate histones *in vitro* with a specific activity about 7.5% of that of insect cell-expressed and affinity-purified FLAG-tagged HDAC1 (typically, 7,500 and 100,000 cpm of [³H]acetate released per nmol of enzyme by the HDAC homologue and FLAG-tagged HDAC1, respectively). The *A. aeolicus* HDAC homologue is inhibited by TSA (half-maximal inhibition at 0.4 μM) and SAHA and binds these inhibitors in a gel-filtration chromatography assay (data not shown). The *in vitro* deacetylase activity of the purified HDAC homologue was observed only after incubation with zinc

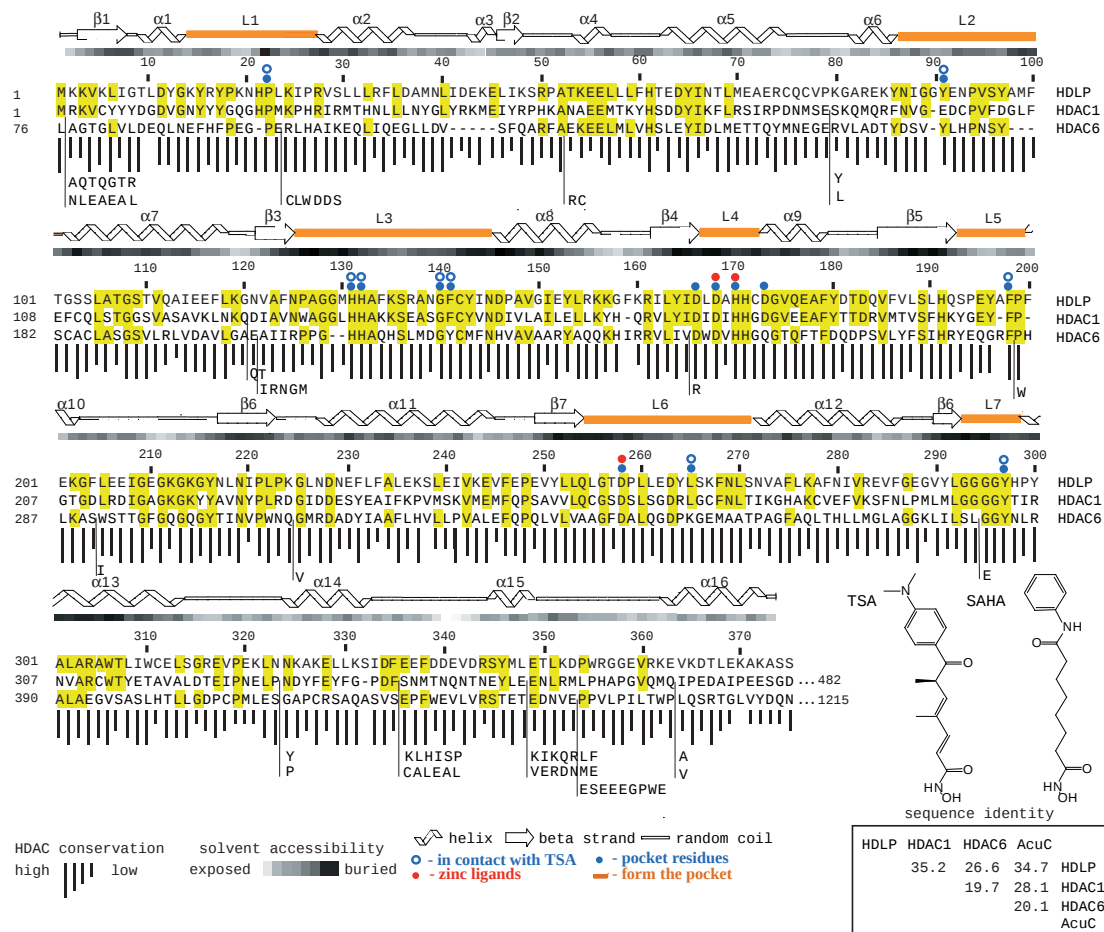


Figure 1 The *A. aeolicus* HDLP has 35.2% identity to human HDAC1. Sequence alignment of HDLP, human class I HDAC1 and class II HDAC6. Residues identical between HDLP and HDAC1 or HDLP and HDAC6 are highlighted in yellow. The bar graph below the sequence alignment indicates the degree of identity among five class I HDAC genes: HDAC1, and its homologues from thale cress, fruit fly, frog and yeast (RPD3). Insertions in HDAC1 and HDAC6 sequences are dropped below the alignment with a vertical line for clarity. Loops that form the active-site pocket are highlighted orange, and residues that make up the pocket walls and active site and those that contact TSA are indicated. The

chemical structures of TSA and SAHA are shown. Class I is characterized by human HDAC1, 2, 3 and yeast RPD3, and class II by human HDAC4, 5, 6 and yeast HDA1 (refs 10–12). The function of HDLP is not known. It has been annotated as *AcuC1* in the genome sequence according to its homology to the *B. subtilis* *AcuC* acetoin utilization protein, whose precise function is also not known. However, the *A. aeolicus* genome appears to lack the *acuA* and *acuB* genes that are part of the *acuABC* operon of *B. subtilis*²⁷, and HDLP is as similar to human HDAC1 (35.2% identity) as it is to *B. subtilis* *AcuC* (34.7% identity).

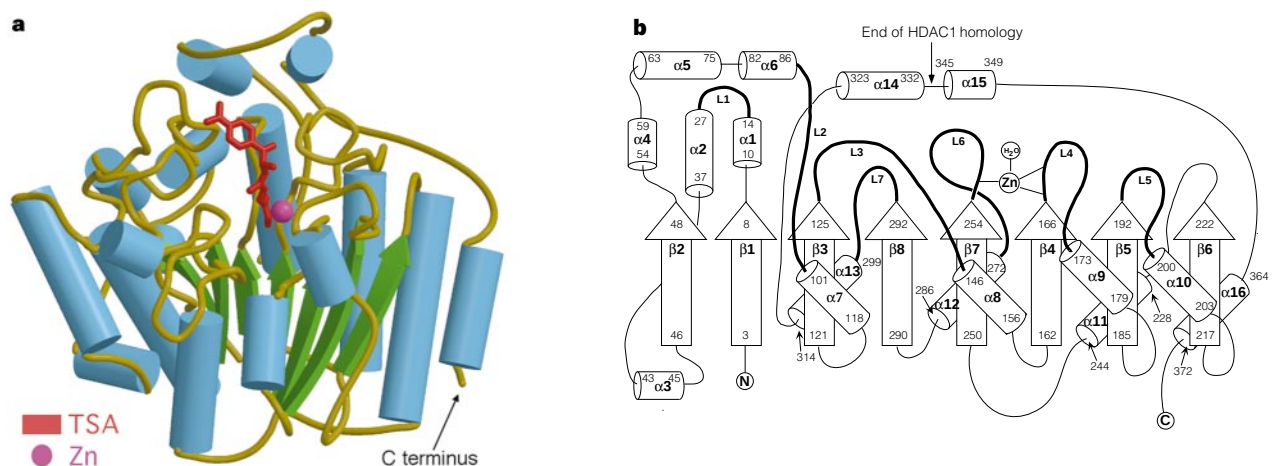


Figure 2 The histone deacetylase catalytic core belongs to the open α/β -family of folds. **a**, HDLP–Zn²⁺–TSA complex. Figure prepared with the programs MOLSCRIPT²⁸ and RASTER3D²⁹. **b**, Topology diagram for HDLP. Loops forming the active-site pocket are in bold. The end of the deacetylase motif is indicated with an arrow. HDLP has the same topology as arginase³⁰, which catalyses the hydrolysis of arginine to ornithine, and the two

structures can be superimposed with a C α r.m.s.d. of 2.5 Å for about 35% of the residues, mapping primarily to the β -sheet. The arginase structure differs in that it has only nine helices instead of sixteen, a two-manganese metal cluster and a wider active site that lacks the internal cavity.

chloride, consistent with previous suggestions that HDAC activity requires a metal cofactor¹⁴, and with our observation that the zinc chelator phenanthroline inhibits HDAC1 (data not shown). No significant activity was observed with HDAC homologue preparations reconstituted with Ca^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} or Mn^{2+} , whereas Co^{2+} , which can often substitute for zinc *in vitro*, produced a comparable level of activity (data not shown). Here we refer to the *A. aeolicus* HDAC homologue as HDLP (histone deacetylase-like protein) to reflect its sequence homology to the HDAC family (Fig. 1), its *in vitro* histone deacetylase activity and its inhibition by the HDAC inhibitors.

The crystal structure reveals that HDLP has a single-domain structure belonging to the open α/β class of folds. It consists of a central eight-stranded parallel β -sheet and sixteen α -helices (Fig. 2). Four helices pack on either face of the β -sheet, forming the core α/β structure characteristic of this class of folds. Most of the remaining eight helices are clustered near one side of the β -sheet (Fig. 2). Large, well-defined loops originate from the carboxy-terminal ends of the β -strands (loops L1–L7; Fig. 2b). The extra helices and the large L1–L7 loops are associated with a significant extension of the structure beyond the core α/β motif. This gives rise to two

prominent architectural features: a deep, narrow pocket and an internal cavity adjacent to the pocket (Fig. 3a).

The pocket has a tube-like shape with a depth of ~ 11 Å. The pocket opening constricts halfway down to ~ 4.5 by 5.5 Å, but becomes wider at the bottom (Fig. 3a). The pocket and its immediate surroundings are made up of seven loops. These originate from strands near the centre of the sheet (L3–L7) and from the additional helices that pack near the ends of the strands (L1 and L2; Figs 1, 2b). The walls of the pocket are covered with hydrophobic and aromatic residues that are identical in HDAC1 (Pro 22, Gly 140, Phe 141, Phe 198, Leu 265 and Tyr 297; Fig. 3b). Of particular significance are Phe 141 and Phe 198, whose phenyl groups face each other in parallel at a distance of 7.5 Å, marking the most slender portion of the pocket (Fig. 3b, c).

In the crystal structure of the zinc-reconstituted enzyme, the zinc ion is positioned near the bottom of the pocket, just beyond its narrowest point. It is coordinated by Asp 168 (O δ 1, 2.1 Å), His 170 (N δ 1, 2.1 Å), Asp 258 (O δ 1, 1.9 Å) and a water molecule (2.5 Å). The protein ligands are arranged in a tetrahedral geometry, but the position of the water molecule, which also hydrogen-bonds to His 131, deviates from this geometry by $\sim 25^\circ$.

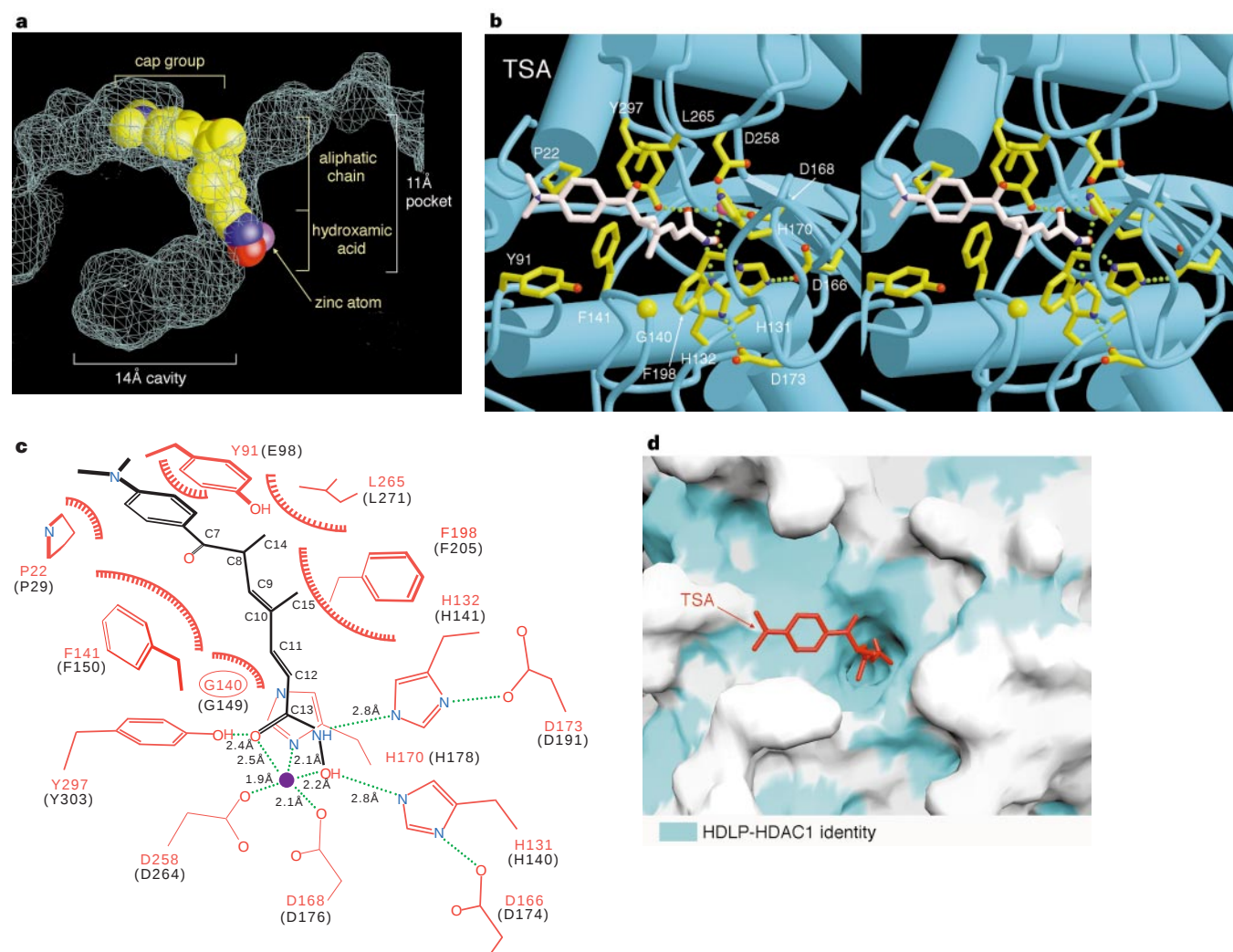


Figure 3 TSA binds inside the pocket making contacts to residues at the rim, walls and bottom of the pocket. **a**, Space-filling representation of TSA in the active-site pocket. The hydroxamic acid group, most of the aliphatic chain and part of the dimethylamino-phenyl group of TSA are buried (60% of TSA's surface area). The internal cavity has a volume of 144 Å³. **b**, Closeup stereo view of the structure of the HDLP–Zn²⁺–TSA complex in an orientation similar to Fig. 2a except for a 90° rotation about the vertical axis. TSA is in white; active-site residues and residues that contact TSA, which are all identical in HDAC1

except Tyr 91, are in yellow. **c**, Schematic representation of HDLP–TSA interactions. TSA is in black and the protein is in red. HDLP residues are labelled in red with their counterparts in HDAC1 indicated in black. Thatched semi-circles indicate van der Waals contacts between hydrophobic protein residues and TSA. Hydrogen bonds are shown as green dashed lines. **d**, Surface representation of the HDLP–TSA interface in a similar orientation to **b**. The protein surface is coloured according to residue identity with HDAC1. Cyan indicates residues that are identical in HDAC1.

In addition to the zinc ligands, the bottom of the pocket contains two histidines (His 131 and His 132), two aspartic acids (Asp 166 and Asp 173) and a tyrosine (Tyr 297), all of which are identical in HDAC1. Each of the histidines makes a hydrogen bond through its N δ 1 to an aspartic acid carboxylate oxygen, with the oxygen located in the plane of the imidazole ring (Fig. 3b, c). This His–Asp arrangement is characteristic of the charge-relay system found in the active sites of serine proteases, where it polarizes the imidazole N ϵ and increases its basicity¹⁵. The Asp 166–His 131 charge-relay pair is positioned deeper inside the pocket, and is more buried than the Asp 173–His 132 charge relay, which is partially solvent exposed. The buried charge relay makes a hydrogen bond (2.6 Å) to the zinc-bound water molecule, which could contribute to the deviation of the water–zinc coordination from ideal geometry. Tyr 297 is positioned next to the zinc, opposite to the two charge-relay systems. Its hydroxyl group lies 4.4 Å from the zinc atom and has no interactions with the rest of the protein (Fig. 3b, c). Next to Tyr 297 there is an opening in the pocket wall, which leads to the adjacent internal cavity.

The internal cavity is made up of portions of the L3 and L7 loops as they emerge from the β -strands and the α 1–L1– α 2 segment. The L1 loop, which demarcates the cavity from the solvent, has higher temperature factors than the rest of the structure, indicating some inherent flexibility that may allow the transient exchange of the cavity contents with the bulk solvent. The cavity is lined primarily with hydrophobic residues and is particularly rich in glycine residues (Fig. 1). There are only two charged residues in the cavity and these are contributed by the L1 loop (Arg 27 and His 21). The function of the cavity is not clear. It is possible that it provides space for the diffusion of the acetate product away from the catalytic centre, which may otherwise be crowded and shielded from the solvent when the substrate is bound.

The HDLP–HDAC1 homology maps mainly to the hydrophobic core and to the L1–L7 loops, with the portions of the loops that make up the pocket and adjacent cavity having the highest level of conservation (Fig. 1). Specifically, all of the polar residues in the active site and the hydrophobic residues that make up the walls of the pocket are identical. Among the residues that make up the internal cavity, the ones closest to the active site are either identical or conservatively substituted (Fig. 1). The HDLP active-site residues that are identical in HDAC1 are also conserved in the class II histone deacetylases. One exception is Asp 173 of the exposed charge-relay system, which is an asparagine in some class II HDACs (Fig. 1). The overall 35.2% HDLP–HDAC1 sequence identity predicts structural similarity with r.m.s. deviation in C α positions of $\sim$ 1.5 Å (ref. 16), and indicates that the 375-amino-acid HDLP protein corresponds to the histone deacetylase catalytic core that is conserved across the HDAC family. The one region likely to have a less similar structure is the 40-residue C terminus of HDLP (335–375), which has significantly lower homology to HDAC1.

In the crystal structure of the HDLP–Zn²⁺–TSA complex, TSA binds by inserting its long aliphatic chain into the HDLP pocket, making multiple contacts to the tube-like hydrophobic portion of the pocket (Fig. 3a). The hydroxamic acid group at one end of the aliphatic chain reaches the polar bottom of the pocket, where it coordinates the zinc in a bidentate fashion and also contacts active-site residues. The aromatic dimethylamino-phenyl group at the other end of the TSA chain makes contacts at the pocket entrance and in an adjacent surface groove, capping the pocket. The length of the aliphatic chain appears to be optimal for spanning the length of the pocket and allowing contacts both at the bottom and at the entrance of the pocket.

The hydroxamic acid coordinates the zinc through its carbonyl (2.4 Å) and hydroxyl groups (2.2 Å), resulting in a penta-coordinated Zn²⁺ (Fig. 3b, c). The hydroxamic acid also hydrogen-bonds with both charge-relay histidines and the Tyr 297 hydroxyl group (Fig. 3b, c) and replaces the zinc-bound water molecule of the active structure with its hydroxyl group. The binding of the TSA hydro-

xamic acid to HDLP shares several features with the binding of hydroxamic-acid-containing inhibitors to zinc metalloproteases, such as neutrophil collagenase¹⁷, collagenase-3 (ref. 18) and thermolysin¹⁹. Like TSA, these inhibitors also (1) coordinate the active-site zinc in a bidentate fashion using their hydroxamate hydroxyl and carbonyl oxygens; (2) replace the nucleophilic water molecule with their hydroxamate hydroxyl groups; and (3) form hydrogen bonds with a general base in the active site.

The five-carbon-long branched aliphatic chain of TSA fits snugly in the narrow portion of the pocket, making multiple van der Waals contacts with all of the hydrophobic groups lining the pocket. Near its centre, the chain contains a methyl-substituted vinyl group that is sandwiched between the phenyl groups of Phe 141 and Phe 98, at the tightest point of the pocket (Fig. 3a–c).

The dimethylamino-phenyl and adjacent carbonyl groups of TSA form a planar structure and contact residues at the rim of the pocket and in an adjacent surface groove (Pro 22, Tyr 91, Phe 141; Fig. 3b, c). This packing is facilitated by the $\sim$ 110° angle in the overall structure of TSA at the junction of the aliphatic chain and the dimethylamino-phenyl group (at C8). Upon TSA binding, the side chain of Tyr 91, which is at the periphery of the pocket and mostly solvent exposed, changes conformation to make space for the dimethylamino-phenyl group. This is the only change near the active site observed upon TSA binding. All but one (Tyr 91) of the HDLP residues that TSA contacts are identical in HDAC1 (Figs 1, 3b, c), indicating that TSA may bind and inhibit HDAC1 in a similar fashion.

SAHA, which has $\sim$ 30-fold weaker inhibitory activity than TSA⁶, binds HDLP similarly (Fig. 4). The SAHA hydroxamic acid group makes the same contacts to the zinc and active-site residues, and the importance of these interactions is underscored by the loss of activity of SAHA derivatives lacking the hydroxamic acid group⁶. The six-carbon-long aliphatic chain of SAHA packs in the tube-like hydrophobic portion of the pocket. Compared to TSA, however, SAHA's aliphatic chain packs less snugly and makes fewer van der Waals contacts, in part because SAHA lacks TSA's C15 methyl-group branch (Figs 3b, 4). SAHA also lacks TSA's double bonds in this region, and this may lead to increased flexibility of the aliphatic chain. The cap group of SAHA consists of a phenyl-amino ketone group. In the crystal structure, the phenyl group has weak electron density, suggesting that it may not pack as well as the cap group of TSA. This could be due, in part, to the larger separation between the hydroxamic and cap groups of SAHA than between those of TSA (Fig. 1).

The natural products trapoxin and HC-toxin, which represent a chemically distinct family of HDAC inhibitors^{4,20}, contain groups that may be analogous to the cap, aliphatic chain and active-site/zinc-binding groups of TSA. These inhibitors do not have a

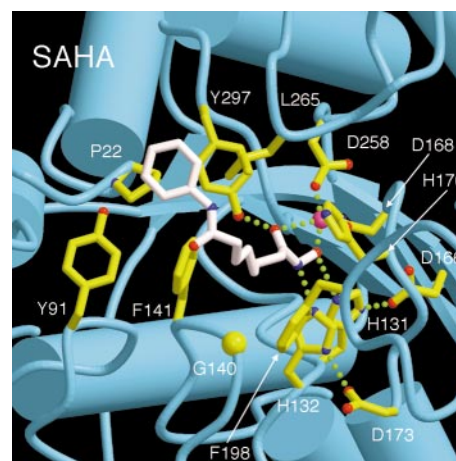


Figure 4 SAHA binds HDLP like TSA, but its aliphatic chain and cap groups make fewer contacts. Close-up view of the HDLP–Zn²⁺–SAHA complex in the same orientation and colour scheme as Fig. 3b. Note that Tyr 91 is not in the TSA-bound conformation.

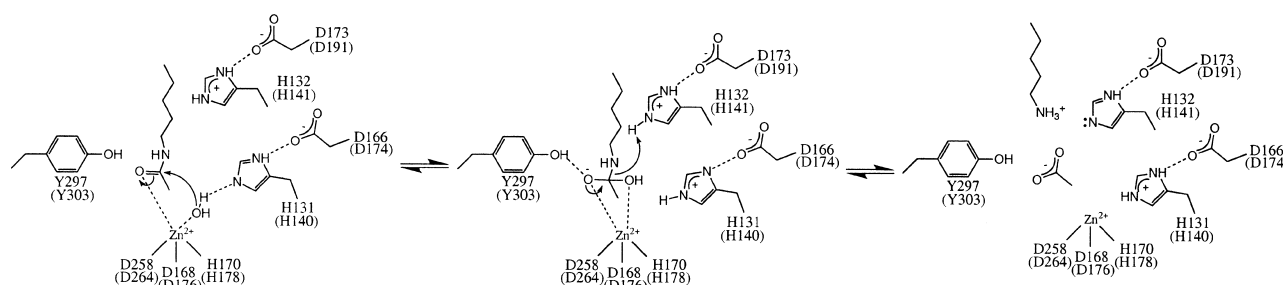


Figure 5 The proposed catalytic mechanism for the deacetylation of acetylated lysine. HDLP active-site residues and their proposed HDAC1 counterparts (in parenthesis) are labelled.

hydroxamic acid, but their epoxy group may crosslink to an active site nucleophile⁴. Also, their ketone group may interact with polar residues, and possibly the zinc, at the bottom of the active-site pocket. This is supported by the observation that the reduction of the carbonyl to a hydroxyl, or its elimination, causes a large decrease in the activity of HC-toxin²⁰. Trapoxin and HC-toxin both have a cyclic tetrapeptide with hydrophobic groups such as L-phenylalanine, D-proline, and L- or D-alanine that could, in principle, serve as a cap. The larger size of their cap group compared to that of TSA might allow them to make more extensive contacts at the rim of the pocket and in the shallow grooves surrounding the pocket entrance (Fig. 3d). The epoxy ketone and cyclic tetrapeptide groups of these inhibitors are linked by a five-carbon aliphatic chain, which probably binds in the hydrophobic tube-like portion of the pocket.

The structures of the HDLP-Zn²⁺-TSA and HDLP-Zn²⁺-SAHA complexes support the overall analogy of the aliphatic chain and hydroxamic acid groups of these inhibitors to the lysine side chain and the acetyl group of the HDAC substrate¹⁰. The branched C8 carbon, where the TSA structure bends as it enters the pocket, may approximate the C α of the lysine amino acid. This positioning of the C α would allow the aliphatic portion of the lysine side chain to span the tube-like portion of the pocket and place the acetylated amino group into the polar bottom of the pocket. The polypeptide backbone could interact with surface residues and in the shallow grooves around the pocket, including the groove where the TSA cap binds (Fig. 3d).

The deacetylase active site has features of both metallo- and serine proteases. We propose a mechanism that has aspects from both families of proteases and that is also consistent with the contacts TSA and SAHA make in the active site (Fig. 5). By analogy to the zinc proteases²¹, the carbonyl oxygen of the N-acetyl amide bond could bind the zinc, and the carbonyl carbon could be positioned in close proximity to the water molecule. The zinc ion could polarize the carbonyl group so that the carbon is a better electrophile, and could also help orient the water molecule. The nucleophilicity of the water molecule would be increased by the negative charge of the buried Asp 166-His 131 charge-relay system to which the water is hydrogen bonded. This is analogous to the activation of a serine hydroxyl by a buried charge-relay system in the serine proteases¹⁵, and to the activation of a water molecule by a glutamic acid in the zinc proteases²¹. The nucleophilic attack of the water on the carbonyl carbon would result in a tetrahedral carbon. This oxy-anion intermediate could be stabilized by two zinc-oxygen interactions, in a manner analogous to the zinc proteases²¹, and possibly by a hydrogen bond from the Tyr 297 hydroxyl group (Fig. 5). In the final step, the carbon-nitrogen bond of the intermediate would break, and the nitrogen of the scissile bond would accept a proton from the exposed Asp 173-His 132 charge relay, yielding the acetate and lysine products (Fig. 5).

The general aspects of this mechanism are supported by mutagenesis studies of *Drosophila* and human HDACs and of HDLP. Mutation of the histidine and aspartic-acid residues of the buried charge-relay system abolished activity^{14,22}, consistent with Asp 166-

His 131 serving as a general base. Mutation of the histidine of the exposed charge relay reduced but did not abolish the activity, consistent with Asp 173-His 132 serving to protonate the epsilon nitrogen atom, and with some class II HDACs having an asparagine instead of aspartic acid in this charge relay. Mutation of Tyr 297 to phenylalanine eliminated the activity of HDLP in our *in vitro* assay (data not shown), supporting a role in catalysis.

The crystal structures of HDLP and the HDLP-Zn²⁺-TSA and HDLP-Zn²⁺-SAHA complexes provide a framework for understanding the catalytic activity and inhibition of the histone deacetylase family, and form a basis for the further development of HDAC inhibitors as antitumour agents. □

Methods

Protein expression and purification

The gene for HDLP (genbank accession no. AE000719) was subcloned from an *A. aeolicus* chromosomal DNA preparation (provided by R. Huber) into the pGEX4T3 vector (Pharmacia). The wild-type HDLP, Cys75Ser/Cys77Ser and active-site mutants were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins, purified by glutathione-sepharose affinity chromatography, cleaved with thrombin and further purified by anion-exchange chromatography. HDLP was reconstituted with Zn²⁺ (we presume the lack of metal in the purified HDLP is due, in part, to the use of DTT during purification) by mixing the Cys75Ser/Cys77Ser double mutant, at 10 mg ml⁻¹, with a 5-fold molar excess of ZnCl₂, followed by fractionation through a G25 desalting column. The use of the Cys75Ser/Cys77Ser mutant largely overcame the problem of protein aggregation during reconstitution, which we presume was due to the binding of free zinc to solvent-exposed cysteines. The HDLP-Zn²⁺-TSA and HDLP-Zn²⁺-SAHA complexes were prepared by mixing the Zn²⁺-reconstituted HDLP Cys75Ser/Cys77Ser double mutant with 1 mM ligand, and were isolated by gel-filtration chromatography. Compared to the apo- or Zn²⁺-HDLP, the concentrated HDLP-Zn²⁺-TSA complex has a lower retention volume in gel-filtration chromatography, and this could correspond to the dimer observed in the crystals of the HDLP-Zn²⁺-TSA complex. Proteins were concentrated to typically 25 mg ml⁻¹ in a buffer of 25 mM bis-tris propane, 500 mM NaCl, 1% isopropanol, pH 7.0 by ultrafiltration. FLAG-epitope-tagged human HDAC1 was overexpressed using a baculovirus expression system in Hi5 (Invitrogen) insect cells grown in suspension in serum-free medium (Sf900, Gibco). The fusion protein was purified by affinity chromatography using Anti-FLAG M2 affinity resin (Sigma) and FLAG Peptide (Sigma).

Crystallization and data collection

We grew crystals of apo-HDLP at room temperature by the hanging-drop vapour-diffusion method, from 7.5% isopropanol, 28% polyethylene glycol (PEG) 1500, 425 mM NaCl, 100 mM Tris-Cl, pH 7.0; they contain one HDLP molecule in the asymmetric unit. Crystals of the HDLP-Zn²⁺ complex were grown from 23% tert-butanol, 27% PEG 1500, 400 mM KCl, 100 mM bis-tris propane-Cl, pH 6.8. Crystals of the HDLP-Zn²⁺-TSA and HDLP-Zn²⁺-SAHA complexes were grown from 23% tert-butanol, 27% PEG 1500, 600 mM KCl, 100 mM bis-tris propane-Cl, pH 6.8, by microseeding. The HDLP-Zn²⁺-TSA crystals contain two complexes in the asymmetric unit. All data were collected on flash-frozen crystals.

MIR analysis, model building and refinement

Heavy-atom soaks were performed with the apo-HDLP crystals in a buffer of 7.5% isopropanol, 30% PEG 1500, 75 mM NaCl, 100 mM Tris-Cl, pH 8.0, supplemented with 1.0 mM thimerosal for 2 h, 5 mM KAu(CN)₂ for 1 h and 1 mM Pb(Me)₃OAc for 2 h. MIR phases, calculated with the program MLPHARE²³ at 2.5 Å, included the anomalous diffraction signal from the thimerosal derivative and had a mean figure of merit of 0.55 (Table 1). The phases were improved by solvent flattening with the program DM²³, and were used to build the initial model with the program O²⁴. The model was refined with the program CNS²⁵.

We determined the structures of the HDLP-Zn²⁺-TSA and HDLP-Zn²⁺-SAHA complexes by molecular replacement with the program AMORE²³ using the apo-HDLP

Table 1 Statistics from the crystallographic analysis

Data set	Native	Hg	Pb	Au	Zn	TSA	SAHA
Space group	C2	C2	C2	C2	C2	$P2_12_12_1$	C2
Resolution (Å)	1.8	2.3	3.5	2.8	2.0	2.1	2.5
Observations	134,952	79,023	11,454	27,722	125,769	180,427	27,568
Unique reflections	32,143	15,958	4,040	8,753	23,643	50,796	11,748
Data coverage (%)	92.3	95.7	86.4	94.3	90.6	93.8	90.6
R_{sym} (%)	2.9	8.4	9.6	8.9	7.2	7.1	8.6
MIR analysis (20.0–2.5 Å):							
Phasing power	—	1.47	1.24	1.10	—	—	—
R_{cullis}	—	0.72	0.78	0.85	—	—	—
$R_{\text{cullis}}(\text{ano})$	—	0.92	—	—	—	—	—

Refinement statistics:

Data set	Resolution (Å)	Reflections ($ F > 1\sigma$)	Total atoms	Water atoms	R -factor (%)	R_{free} (%)	Bonds (Å)	Angles (°)	B -factor (Å ²)
HDLP	1.8	31,550	3,214	228	19.8	24.0	0.010	1.63	3.55
HDLP–Zn	2.0	23,582	3,424	434	22.0	25.8	0.009	1.48	1.04
HDLP–Zn–TSA	2.1	44,122	6,475	456	22.4	25.8	0.008	1.78	3.83
HDLP–Zn–SAHA	2.5	11,113	3,152	144	20.0	27.7	0.009	1.58	1.12

$R_{\text{sym}} = \sum_i \sum_j |I_{ij} - \langle I_i \rangle| / \sum_i \sum_j I_{ij}$ for the intensity (I) of i observations of reflection h . Phasing power is $(F_o)/E$, where (F_o) is the root-mean-square heavy atom structure factor and E is the residual lack of closure error. R_{cullis} is the mean residual lack of closure error divided by the dispersive difference. R -factor = $\sum |F_{\text{obs}} - F_{\text{calc}}| / \sum |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. Figure of merit = $|F(hkl)_{\text{best}}| / F(hkl)$. R_{free} is R -factor calculated using 5% of the reflection data chosen randomly and omitted from the start of refinement. r.m.s.d.: root mean square deviations from ideal geometry and root mean square variation in the B -factor of bonded atoms.

structure as a search model. The structure of TSA from the Cambridge Structural Database (Refcode TRCHST) was used to define stereochemical restraints used in the refinement with the program CNS. The restraints for SAHA were constructed based on stereochemical parameters from TSA and amino acids. The dimer interface in the HDLP–Zn²⁺–TSA crystals primarily involves Phe 200 and Tyr 91 on the protein surface. These two residues are not conserved in HDAC1.

Histone deacetylase assays.

We assayed purified proteins by incubating them with 10 µg of [³H]acetyl-labelled murine erythroleukaemia cell-derived histone substrate for 30–60 min at 37 °C in a total volume of 30 µl as described²⁶. The final concentrations of HDLP–Zn²⁺ and FLAG-tagged HDAC1 were 3.6 µM and 0.24 µM, respectively, and assays were performed in duplicate. Inhibitors were added in the absence of substrate and incubated on ice for 20 min, substrate was added, and the assay performed as described above. Dialysis of FLAG-tagged HDAC1 against 20 µM ZnCl₂ had no significant effect on its activity, suggesting that FLAG-tagged HDAC1 retains a metal as purified.

Received 14 June; accepted 30 July 1999.

- Davie, J. R. & Chadee, D. N. Regulation and regulatory parameters of histone modifications. *J. Cell Biochem. (Suppl.)* **30–31**, 203–213 (1998).
- Kouzarides, T. Histone acetylases and deacetylases in cell proliferation. *Curr. Opin. Genet. Dev.* **9**, 40–84 (1999).
- Fenrick, R. & Hiebert, S. W. Role of histone deacetylases in acute leukemia. *J. Cell. Biochem. (Suppl.)* **30–31**, 194–202 (1998).
- Yoshida, M., Horinouchi, S. & Beppu, T. Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. *Bioessays* **17**, 423–430 (1995).
- Richon, V. M. *et al.* Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. *Proc. Natl Acad. Sci. USA* **93**, 5705–5708 (1996).
- Richon, V. M. *et al.* A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc. Natl Acad. Sci. USA* **95**, 3003–3007 (1998).
- Cohen, L. A. *et al.* Inhibition of N-methylnitrosourea-induced tumours by the cytodifferentiating agent, suberanilohydroxamic acid (SAHA). *Proc. AACR* **39**, 108, abstr. 736 (1998).
- Desai, D., El-Bayoumy, K. & Amin, S. Chemopreventive efficacy of suberanilohydroxamic acid (SAHA), a cytodifferentiating agent, against tobacco-specific nitrosamine 4-(methylnitroso-amino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in female A/J mice. *Proc. AACR* **40**, 2396, abstr. 362 (1999).
- Struhl, K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.* **12**, 599–606 (1998).
- Taunton, J., Hassig, C. A. & Schreiber, S. L. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science* **272**, 408–411 (1996).
- Grozinger, C. M., Hassig, C. A. & Schreiber, S. L. Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proc. Natl Acad. Sci. USA* **96**, 4868–4873 (1999).
- Fischle, W. *et al.* A new family of human histone deacetylases related to *Saccharomyces cerevisiae* Hda1p. *J. Biol. Chem.* **274**, 11713–11720 (1999).
- Leipe, D. D. & Landsman, D. Histone deacetylases, acetoin utilization proteins and acetylpolymine amidohydrolases are members of an ancient protein superfamily. *Nucleic Acids Res.* **25**, 3693–3697 (1997).

- Hassig, C. A. *et al.* A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. *Proc. Natl Acad. Sci. USA* **95**, 3519–3524 (1998).
- Fersht, A. R. & Sperling, J. The charge relay system in chymotrypsin and chymotrypsinogen. *J. Mol. Biol.* **74**, 137–149 (1973).
- Chothia, C. & Lesk, A. M. The relation between the divergence of sequence and structure in proteins. *EMBO J.* **5**, 823–826 (1986).
- Grams, F. *et al.* Structure determination and analysis of human neutrophil collagenase complexed with a hydroxamate inhibitor. *Biochemistry* **34**, 14012–14020 (1995).
- Lovejoy, B. *et al.* Crystal structures of MMP-1 and -13 reveal the structural basis for selectivity of collagenase inhibitors. *Nature Struct. Biol.* **6**, 217–221 (1999).
- Holmes, M. A. & Matthews, B. W. Binding of hydroxamic acid inhibitors to crystalline thermolysin suggests a pentacoordinate zinc intermediate in catalysis. *Biochemistry* **20**, 6912–6920 (1981).
- Shute, R. E., Dunlap, B. & Rich, D. H. Analogues of the cytostatic and antimetogenic agents chlamydocin and HC-toxin: synthesis and biological activity of chloromethyl ketone and diazomethyl ketone functionalized cyclic tetrapeptides. *J. Med. Chem.* **30**, 71–78 (1987).
- Christianson, D. W. & Lipscomb, W. N. Carboxypeptidase A. *Acc. Chem. Res.* **22**, 62–69 (1989).
- Kadosh, D. & Struhl, K. Histone deacetylase activity of Rpd3 is important for transcriptional repression *in vivo*. *Genes Dev.* **12**, 797–805 (1998).
- The CCP4 suite: Programs for computational crystallography. *Acta Crystallogr. D* **50**, 760–763 (1994).
- Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A* **47**, 110–119 (1991).
- Brunger, A. T. *et al.* Crystallography and NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D* **54**, 905–921 (1998).
- Hendzel, M. J., Delcuve, G. P. & Davie, J. R. Histone deacetylase is a component of the internal nuclear matrix. *J. Biol. Chem.* **266**, 21936–21942 (1991).
- Deckert, G. *et al.* The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* **392**, 353–358 (1998).
- Kraulis, P. Molscript: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950 (1991).
- Merritt, E. A. & Bacon, D. J. Raster3D-Photorealistic molecular graphics. *Methods Enzymol.* **277**, 505–524 (1997).
- Kanyo, Z. F., Scholnick, L. R., Ash, D. E. & Christianson, D. W. Structure of a unique binuclear manganese cluster in arginase. *Nature* **383**, 554–557 (1996).

Acknowledgements

We thank R. Huber for the *A. aeolicus* chromosomal DNA preparation; C. A. Hassig and S. L. Schreiber for the HDAC1-FLAG baculovirus and for helpful discussions; A. S. Mildvan and J. Thomson for advice on the reaction mechanism; and C. Murray for administrative help. Supported by the Howard Hughes Medical Institute, the NIH, the Dewitt Wallace Foundation and the Samuel and May Rudin Foundation.

Correspondence and requests for materials should be addressed to N.P.P. (e-mail: nikola@xray2.mskcc.org). Coordinates have been deposited with the Brookhaven Protein Data Bank (accession numbers 1C3P, 1C3R and 1C3S for HDLP, HDLP–Zn–TSA and HDLP–Zn–SAHA, respectively).